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13. ABSTRACT *(Maximum 200 Words)*
This work has been directed towards developing an understanding of the molecular and signal-transduction events mediating growth arrest and cell death in breast tumor cells after exposure to ionizing radiation. Our findings that the breast tumor cell fails to undergo apoptotic cell death in response to irradiation (as well as in response to Adriamycin) have provided the incentive for developing approaches for radiosensitization (and chemosensitization) of the breast tumor cell by combining conventional chemotehrapy and radiotherapy with relatively nontoxic Vitamin D3 analogs. In addition, we have discovered that irradiation has the capacity to promote the uptake and expression of exogenous genes, a finding which is the basis for the development of strategies for the delivery of cytotoxic and apoptosis-promoting genes to both p53 wild-type and p53 mutated breast tumor cells. Finally, the possible role of p53 in enforcing the fidelity of double-strand break repair has been investigated in matched p53+ and p53-defective breast epithelial cells. Putative double-strand break misrepair events, induced by bleomycin and detected as *HPRT* mutations, have been analyzed at both the chromosomal level and the DNA sequence level. Chromosomal stability, delayed reproductive death, and radiation-induced apoptosis and cell cycle perturbations have been assessed in the mutant cells in an attempt to determine whether specific types of misrepair events were accompanied by changes in these responses.

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INTRODUCTION *Subject and scope of research* This work is directed towards developing an understanding of the molecular and signal-transduction events mediating growth arrest and cell death in breast tumor cells after exposure to ionizing radiation. Our studies have been focused primarily on the p53, $p21^{waf1/cip1}$, Myc and E2F-1 proteins; these proteins have overlapping and possibly mutually exclusive functions in the regulation of cell growth and apoptotic cell death pathways in response to DNA damage. Our findings that the breast tumor cell fails to undergo apoptotic cell death in response to irradiation (as well as in response to Adriamycin) have provided the incentive for developing approaches for radiosensitization (and chemosensitization) of the breast tumor cell. In addition, we have discovered that irradiation has the capacity to promote the uptake and expression of exogenous genes, a finding which is the basis for the development of strategies for the delivery of cytotoxic and apoptosis-promoting genes to both p53 wild-type and p53 mutated breast tumor cells. Finally, the possible role of p53 in enforcing the fidelity of double-strand break repair has been investigated in matched p53+ and p53-defective breast epithelial cells. Putative double-strand break misrepair events, induced by bleomycin and detected as *HPRT* mutations, have been analyzed at both the chromosomal level and the DNA sequence level. Chromosomal stability and cell cycle perturbations have been assessed in both the treated cell cultures as a whole and in selected mutant cell clones, in an attempt to determine whether specific types of misrepair events were accompanied by changes in these parameters.

Background While radiation therapy and chemotherapy using the drug Adriamycin are effectively utilized in the management of breast cancer, the recurrence of disease indicates the limitations of these treatment protocols. We believe that breast tumor cells may demonstrate primary resistance to radiotherapy (and to chemotherapy), in part through their refractoriness to the induction of apoptotic cell death. Furthermore, even in breast tumor cells which are initially responsive to radiotherapy and chemotherapy, the absence of apoptotic cell death may permit the acquisition of a radioresistant and chemoresistant phenotypes during the course of treatment -leading to recovery of proliferative capacity in tumor cell subpopulations.

An extensive literature describes the closely-linked signal transduction pathways which mediate growth arrest and/or cell death in cells which incur DNA damage by irradiation or drugs such as Adriamycin As shown in **Figure 1**, irradiation, as well as other modalities which induce DNA damage are known to up-regulate levels of the tumor suppressor protein, p53 (Kuerbitz et al, 1992; Zhan et al, 1993; Dulic et al, 1994; Gudas et al, 1995), which in turn increases levels of the cyclin-dependent kinase inhibitory protein, $p21^{waf1/cip1}$ (Di Leonardo et al, 1994; Dulic et al, 1994; Bae et al, 1995; Gudas et al, 1995). Inhibition of cyclin dependent kinases results in abrogation of the phosphorylation of the tumor suppressor protein, Rb (Nigg et al, 1995; Dimri et al, 1996) - which then binds to and inactivates the transcription factor, E2F (Chellappan et al, 1991; Hiebert et al, 1992; Almasan et al, 1995; Weinberg et al, 1995). E2F is thought to regulate the expression of a spectrum of genes associated with DNA synthesis including c-*myc*, DNA polymerase alpha, thymidine kinase and thymidine synthetase (Almasan et al, 1995; Martin et al 1995). Interference with E2F function is postulated to block DNA synthesis and promote growth arrest (Johnson et al, 1993; Almasan et al, 1995).

While the p53, Myc and E2F proteins are fundamental components of the G_1 cell cycle checkpoint, all of these proteins have also been shown to mediate apoptosis or programmed cell death in a variety of tumor cell models in response to DNA damage (Evan et al, 1992; Almasan et al, 1995; Henneking et al, 1995; Lowe et al, 1995). Conversely, up regulation of $p21^{waf1/cip1}$ in response to DNA damage is though to abrogate the apoptotic pathway (Lin and Benchimol, 1995: Attardi et al, 1996). Although many types of DNA damage can cause an increase in p53 levels and activate the cascade described above, this pathway appears to be particularly sensitive to double strand breaks. Indeed, transfection experiments have suggested that the presence of one double-strand break in a cell nucleus, even on a nonessential plasmid, can activate a p53-dependent checkpoint and arrest the cell in G_1 (Huang et al, 1996). In addition, the enhanced apoptotic responses of cells with defective double-strand break repair suggest that double-strand breaks may be the critical triggering lesion for radiation-induced apoptosis as well (Meng et al., 1998; Nussenzweig et al., 1997). Thus, the upstream events in radiation-induced G_1 arrest and apoptotic cell death may be intimately linked to the recognition and processing of double-strand breaks. Moreover, the possible implication of the *BRCA1* and *BRCA2* (hereditary breast cancer) gene products in double-strand break repair, by virtue of their association with the known repair factor hRad51 in nuclear foci in irradiated cells (Bishop et al., 1998; Chen et al., 1999), may suggest a specific link between double-strand break repair and breast cancer.

Purpose The goal of these studies has been to understand the role of c-*myc* and the p53 protein in the pathway leading to growth arrest in the breast tumor cell. As indicated in the body of this report, we have made significant progress relating to this component of the proposal. In the course of this work, we have concluded that the relative refractoriness of breast tumor cells to the induction of apoptotic cell death in response to radiation or chemotherapeutic agents which induce DNA damage represents an observation with potentially important clinical ramifications. Consequently, we have extended our efforts to develop approaches for the promotion of apoptotic cell death in both p53 wild-type and p53 mutated breast tumor cells. An additional component of this work was to investigate the repair of free radical-mediated double-strand breaks (using bleomycin as a model radiomimetic agent) in breast epithelial cells having wild-type versus mutant p53 genes, and the possible relationship of double-strand break repair and repair fidelity to chromosomal stability and cell death.

BODY

Four of the six specific aims composing this grant relate to the suppression of c-myc expression and Myc protein levels in the response of the breast tumor cells to radiation and the linkage of alterations in c-myc expression and function to upstream regulators of the DNA damage response pathway (p53 and $p21^{waf1/cip1}$). In order to evaluate the general applicability of the data generated with ionizing radiation, we have extended this work to one of the primary drugs utilized in the treatment of breast cancer, the anthracycline antibiotic, Adriamycin. We have established that the breast tumor cell is relatively refractory to apoptosis (programmed cell death) in response to both radiation and Adriamycin. Consequently, we have further modified the direction of our research to develop approaches for sensitizing the breast tumor cell to Adriamycin and radiation through the promotion of apoptotic cell death.

In the fifth specific aim, as revised in a previous annual report, we proposed to compare the frequency and molecular nature of both small deletions and gene rearrangements induced by bleomycin in 184B5 ($p53^{+}$) and 184B5-E6*tfx*C6 (p53-) cells. In the sixth specific aim (also revised), we proposed to determine whether gene rearrangements in the two cell lines are accompanied by (1) translocations specifically involving the X chromosome, (2) global chromosomal instability, (3) changes in radiation-induced cell cycle perturbations, (4) apoptosis and (5) delayed reproductive death.

Technical Objective 1: To test the hypothesis that ionizing radiation suppresses the expression of the oncogene, c-myc, in select breast tumor cell lines and that radiation-indued suppression of c-myc expression is a downstream event related to the induction of p53 and/or $p21^{waf1/cip1}$.

Technical Objective 4: To test the hypothesis that suppression of c-myc expression and Myc protein activity are, in part, responsible for the relative refractoriness of the breast tumor cell to apoptotic cell death.

Tasks associated with Technical Objectives 1 and 4 which have been completed:

Task 1: Growth inhibition assays in breast tumor cell lines

Task 2: Northern analysis in breast tumor cell lines of c-myc, p21 and GAPDH.

Task 3: Western analysis of p53 levels in response to ionizing radiation. Time and dose dependence.

Task 4: Assessment of cell death in irradiated cells.

Task 5: Assessment of apoptosis in irradiated cells.

We initially addressed the relationship between p53 and/or $p21^{waf1}$ and c-myc expression by determining the influence of ionizing radiation on c-myc expression in the p53 wild-type MCF-7 and p53 mutant MDA-MB231 breast tumor cell lines. As indicated in Figures 6 and 7 of Manuscript # 1, radiation produced a time and dose-dependent suppression of c-myc expression in the MCF-7 cells; in contrast, in MDA-MB231 breast tumor cells (Figures 8 and 9 of manuscript # 1) the suppression of c-myc expression was minimal (between 20-% to 30%) and was not dose-dependent. Figure 10 in manuscript # 1 indicates that the extent of suppression of c-myc expression was predictive of the extent of growth arrest (measured 72 hours later). We further found that ionizing radiation failed to induce apoptotic cell death in these cells based on the absence of DNA fragmentation using the TUNEL assay (Figure 5 in Manuscript # 1) and the lack of morphological changes such as cell shrinkage and chromosomal condensation (Figure 4 in Manuscript # 1). Rather than cell death, the primary response to ionizing radiation was a prolonged growth arrest (Figure 3 in Manuscript #1). Interestingly, sensitivity to ionizing radiation was essentially identical in the p53 wild-type and p53 mutant breast tumor cell lines (Figure #1 in Manuscript 1). This latter observation suggested that the suppression of c-myc expression is unlikely to be a critical factor in the susceptibility of the breast tumor cell to ionizing radiation-induced cell killing.

<u>Technical Objective 2:</u> To determine whether suppressed c-myc expression is required for growth arrest in breast tumor cells or simply reflects alterations in the growth regulatory pathway.

<u>Technical Objective 3:</u> To examine the hypothesis that ionizing radiation influences the level, stability and activity of the Myc protein in breast tumor cells.

Tasks associated with Technical Objectives 2 and 3 which have been deferred and which are currently in progress.

Objective 2, Task 1: Develop c-myc transfectants.

Objective 2, Task 2: Analysis of transfectants.

Objective 2, Task 3: Determination of radiosensitivity of transfectants.

Objective 3, Task 2: Determination of Myc protein stability.

Objective 3, Task 3: Determination of Myc protein activity in irradiated cells using the ODC-CAT assay and gel shift analysis.

Tasks associated with Technical Objective 3 which have been completed.

Task 1: Determination of radiation effects on Myc protein levels by Western analysis as a function of time and dose.

As indicated above, a number of tasks associated with Objectives 2 and 3 have been delayed. The basis for delaying these tasks relates to our findings which strongly suggest that c-myc expression is unlikely to be a critical element in the response to ionizing radiation. As indicated above, sensitivity to ionizing radiation was similar in MCF-7 and MDA-MB231 breast tumor cells despite the minimal effects of radiation on c-myc expression in the MDA-MB231 cells. Furthermore, in attempting to extend this work to another p53 wild-type breast tumor cell line, ZR-75 cells, we discovered that while ionizing radiation induces p53 and $p21^{waf1}$ in these as well as MCF-7 cells

(Figure 2), there was no evident suppression of c-myc expression in the ZR-75-1 cell line. As both MCF-7 and ZR-75 breast tumor cells demonstrate a similar pattern of growth arrest after irradiation, these studies, taken together with the findings in MDA-MB231 cells, tend to argue against the involvement of c-myc in radiation-induced growth arrest.

Our renewed interest in pursuing the role of c-myc in the response of the breast tumor cell to both Adriamycin and ionizing radiation is based on our recent findings relating to induction of replicative senescence as described below.

Technical Objective 3: To examine the hypothesis that ionizing radiation influences the level, stability and activity of the Myc protein in breast tumor cells.

Task 4: Development of p21 transfectants.

Task 5: Determination of the influence of dysregulated p21 on the induction of apoptosis by ionizing radiation.

It has been reported that increases in levels of $p21^{waf1/cip1}$ are antagonistic to the apoptotic pathway (Lin and Benchimol, 1995; Attardi et al, 1996). We find that radiation as well as adriamycin produce profound increased in $p21^{waf1/cip1}$ levels in the p53 wild-type cells **(Figure 2)**. In order to test the hypothesis that abrogation of $p21^{waf1/cip1}$ induction confers susceptibility to apoptosis, we have been working to transfect MCF-7 breast cancer cells with a $p21^{CIP1}$ antisense construct. Such a construct has been found to sensitize p53 null human leukemia cells (e.g., U937) to apoptosis induced by both the antimetabolite ara-C as well as to ionizing radiation in Dr. Steven Grant's laboratory (Freemerman et al, 1997). We hypothesized that dysregulation of this cyclin-dependent kinase inhibitor would interfere with the G_1/S and/or G_2M checkpoint machinery, and, in so doing, lower the threshold for IR-mediated lethality.

To test this hypothesis, multiple attempts were made to stably transfect MCF-7 cells with the $p21^{CIP1}$ antisense construct in a pcDNA 3.1 vector containing a hygromycin resistance marker. However, despite isolating and characterizing over 40 individual surviving clones, a clear reduction in $p21^{CIP1}$ induction by either IR or doxorubicin could not be demonstrated. Subsequently, in conjunction with the Radiation Oncology Shared Adenovirus Facility, and in collaboration with Dr. Kristoffer Valerie, director of the facility, an adenoviral strategy was employed to transfect MCF-7 cells with the construct. Despite numerous attempts, and evaluation of multiple clones, inhibition of $p21^{CIP1}$ induction following treatment with IR (e.g., 1-5 Gy) or doxorubicin could not be achieved. An example of these efforts is presented in **Figure 3**, which demonstrates induction of p21 by ionizing radiation even in the p21 antisense cells. .

Insights into this problem emerged from studies involving a U937 cell line which had been stably transfected with a temperature-sensitive p53 construct (Vrana et al., 1998). These cells express wild-type p53 at the permissive temperature (32^0 C) but not at 37^0 C. Interestingly, at the permissive temperature, constitutive expression of $p21^{CIP1}$ is noted, and enhanced induction $p21^{CIP1}$ to response to various stimuli is observed. It was found that transfection of these cells with the $p21^{CIP1}$ antisense construct was unable to block $p21^{CIP1}$ induction at the permissive temperature (e.g., when p53 is active). This stands in contrast to U937 wild-type cells, which are p53 null. Together, these findings suggest that p53 dependent $p21^{CIP1}$ induction which is operative in MCF-7 cells and in U937 cell transfectants at the permissive temperature, does not permit suppression of $p21^{CIP1}$ expression in response to stimuli such as IR or doxorubicin. This would explain the inability of the $p21^{CIP1}$ antisense construct to operate in MCF-7 cells and in U937 cells expressing wild-type p53.

In view of evidence that inhibitors of the MEK/MAPK pathway, which lies upstream of $p21^{CIP1}$, can enhance the radiosensitivity of leukemic cells (Cartee et al., 2000), studies were undertaken to examine the effects of the pharmacological MEK inhibitors such as PD98059 on the radiosensitivity of MCF-7 cells. As shown in **Figure 4**, blockade of MAP kinase by PD98059 also blocked the induction of $p21^{waf1/cip1}$ by ionizing radiation. However, there was no evidence of a significant increase in apoptosis or growth inhibition. Consequently, we tentatively conclude that abrogation of $p21^{waf1/cip1}$ induction does not of itself confer sensitivity to irradiation or promote apoptotic cell death in the breast tumor cell.

Basis for renewed interest in c-myc function in growth arrest and cell death in response to ionizing radiation (and adriamycin) in the breast tumor cell.

In studying the interaction of both ionizing radiation and the antitumor drug, Adriamycin, with the breast tumor cell, we have discovered that both of these modalities induce replicative senescence in the MCF-7 breast tumor cell line (Di et al); in contrast, in the MDA-MB231 cells, after a period of prolonged growth arrest, a wave of delayed apoptosis is evident. **Figure 5** demonstrates that Adriamycin promotes beta galactoside expression, a marker of replicative senescence (Dimri et al) in the MCF-7 cells but not in the p53 mutant MDA-MB231 cells. **Figure 6** demonstrates that Adriamycin induces down-regulation of hTERT, the catalytic subunit of telomerase in the MCF-7 cells as well as suppressing telomerase activity. **Figure 7** prevents the differential responses of the MCF-7 and MDA-MB231 breast tumor cells to Adriamycin. While the MCF-7 cells undergo prolonged growth arrest, with eventual recovery of proliferative function in a cell subpopulation, the MDA-MB231 cells undergo a delayed apoptosis, which is evident based on terminal transferase end-labeling of the DNA (TUNEL assay) shown in **Figure 8.** Similar effects (down-regulation of hTERT and telomerase activity and promotion of senescence) were evident in MCF-7 cells exposed to ionizing radiation, while the MDA-MB231 cells also demonstrated delayed apoptosis (not shown); but neither Adriamycin nor ionizing radiation suppressed hTERT expression or telomerase in the MDA-MB231 cells (not shown).

We believe that the suppression of c-myc expression which we have identified previously in response to both Adriamycin and ionizing radiation (Fornari et al, 1996; Watson et al, 1997) could be the basis for the observed effects on the expression of hTERT and telomerase activity. This hypothesis is based on the findings that c-myc has been shown a direct regulator of hTERT expression (Greenberg et al, 1999; Wang et al, 1999, Kyo et al, 2000). Consequently, we are currently in the process of developing both p53 wild-type and p53 mutant breast tumor cells with inducible expression of either c-myc or a dominant negative c-myc in order to examine the linkage between regulation of c-myc, the senescence pathway and growth arrest. Therefore, Tasks 1, 2 and 3 associated with Technical Objective 2 and Tasks 2 and 3 associated with Technical Objective 3 are currently being pursued in this laboratory within the context of replicative senescence in the breast tumor cell.

Additional Findings/Alternative Approaches: Radiosensitization and chemosensitization of the breast tumor cell; promotion of apoptosis by exposure of cells to Vitamin D3 and the hypocalcemic Vitamin D3 analogs EB 1089 and ILX-23-7553.

We have established that ionizing radiation and adriamycin fails to promote apoptotic cell death in the breast tumor cell (Fornari et al, 1996; Watson et al, 1997). In work supported by this grant, we have determined that the Vitamin D3 analog EB 1089 enhances sensitivity to Adriamycin, shifting the dose response curve so that a 50% reduction in clonogenic survival is evident at 5nM rather than 30nM (Figure 2 of Manuscript # 2). The inclusion of EB 1089 with adriamycin also confers susceptibility to adriamycin-induced apoptosis (Figure 4 of Manuscript # 2). Interestingly, the combination of EB 1089 with adriamycin does not appear to be particularly effective in p53 mutant cells (Figure 4 of Manuscript #2). A similar pattern of responses (enhancement of sensitivity, induction of apoptosis and preferential interactions in p53 wild-type cells) was evident when combining EB 1089 with ionizing radiation (Sundaram and Gewirtz, 1999)) and when utilizing another Vitamin D3 analog, ILX-23-7553 with either adriamycin or radiation (Chaudhry et al, in press). These studies suggest that the Vitamin D analogs (which are relatively nontoxic compounds) have the potential to enhance the effectiveness of radiotherapy and chemotherapy in the clinical treatment of breast cancer.

<u>Technical Objective 5:</u> In the (revised) fifth technical objective, we proposed to compare the frequency and molecular nature of both small deletions and gene rearrangements induced by bleomycin in 184B5 ($p53^+$) and 184B5-E6*tfx*C6 (p53-) cells.

<u>Technical Objective 6:</u> In the (revised) technical objective, we proposed to determine whether gene rearrangements in the two cell lines are accompanied by (1) translocations specifically involving the X chromosome, (2) global chromosomal instability, (3) changes in radiation-induced cell cycle perturbations, (4) apoptosis and (5) delayed reproductive death.

Task 6: Screening of breast tumor cell lines for functional *HPRT* hemizygosity and mutability by ionizing radiation.

In the first year of the project, four mammary epithelial cell lines were evaluated for use in mutagenesis studies: MCF-7, ZR-75, MCF-10A and 184B5. ZR-75 was excluded because it was tetraploid, and MCF-7 was excluded because it had two active X chromosomes. MCF-10A was found to have a relatively low plating efficiency, which would make isolation of individual mutant clones difficult. The mammary epithelial line 184B5, however, had reasonable plating efficiency. Moreover, the availability of an existing p53-defective derivative, 184B5-E6*tfx*c6 , made this line ideal for studies of the possible effect of p53 status on cellular response to double-strand breaks (DSBs) In 184B5-E6*tfx*C6 cells (Gudas et al., 1995), p53 function is abrogated by a expression of a stably transfected human pappiloma virus E6 gene. Preliminary experiments showed that 184B5 cells could be arrested in G_0 phase by growth to confluence followed by partial growth factor deprivation. This protocol reduced the S-phase fraction from 13% to 1.3%, as determined by double-label flow cytometry (aminoactinomycin for DNA content and bromodeoxyuridine for DNA synthesis). In preliminary experiments, treatment of these growth arrested cells with the radiomimetic drug bleomycin at low doses for two days increased the mutation frequency approximately tenfold. Subsequent experiments showed that 184B5-E6*tfx*C6 cells could also be arrested in G_0 (S-phase fraction reduced from 13.7% to 5.1%) and were about as mutable by bleomycin as were the parental cells. Before proceeding with comparative analysis of the induced mutants in the two cell lines, however, it was important to verify the abrogation of normal p53 function in the 184B5-E6*tfx*C6 cell line. As expected, Western blotting showed that 184B5 cells contained detectable levels of p53 in normal growth, and the level was markedly elevated for several hours following γ-irradiation. In contrast, p53 was undetectable in 184B5-E6*tfx*C6 cells either with or without irradiation (**Figure 9**).

In order to determine whether the apparent absence of p53 protein was accompanied by the expected loss of G_1 arrest, the effect of γ-irradiation on the cell cycle distribution was examined in both cell lines by flow cytometry. The cell cycle distribution as determined by propidium iodide staining and DNA flow cytometry is shown in **Figures 10 and 11**. The expected normal cell cycle distribution was observed in control cell cultures from both cell lines (**Figures 10A and 11A**). After cells were exposed to 6 Gy ^{60}Co γ-rays, a substantial fraction of cells were blocked in G_2/M phase, with the G_2/M fraction increasing 23% to 40% in the 184B5 cell line (**Figure 10C**) and from 32% to 57% in the 184B5-E6*tfx*C6 cell line (**Figure 11C**). This is consistent with the notion that irradiation induced a transient division delay which could include a G_1 arrest, an S-phase delay and a G_2 arrest (Hartwell and Kastan, 1994). In order to determine the fraction of irradiated cells that was arrested in G_1 phase, the microtubule inhibitor nocodazole was used arrest cells in mitosis and prevent any cells from entering a new round of cell division following irradiation. After treatment with nocodazole alone, the majority of the cells were blocked in G_2/M phase in both cell lines (**Figures 10B and 11B**). When nocodazole was added for 12 hr after irradiation, the 184B5 cell line was arrested in G_0/G_1 phase, as demonstrated by the cell population shifting from G_2/M to G_0/G_1 (**Figure 10D**), while in the 184B5-E6*tfx*C6 cell lines (**Figure 11D**) irradiated and unirradiated cells showed nearly identical profiles with a large majority of cells blocked in G_2. These results indicated that the 184B5 cell line has normal p53 function and an intact DNA damage-dependent G_0/G_1 arrest pathway. In contrast, 184B5-E6*tfx*C6 cell line has defective p53 function and has lost the G_0/G_1 checkpoint in response to DNA damage. The percentage of the cell population in each phase of the cell cycle under different treatment condition is summarized in **Table 1**.

Task 7: Generation of HPRT mutants and screening by Northern blot

Before proceeding with mutagenesis experiments, several survival assays were performed with both cell lines. From these studies, doses of 2.5 and 5 μg/ml, giving survival of about 40% and 30%, respectively, were chosen for mutagenesis studies.

For each mutagenesis assay, six independent, actively growing cultures from each of the two cell lines were plated in medium containing hypoxanthine, amethopterin and thymidine (HAT) for approximately 48 hr before the cells reached confluence. The cell cultures were then fed fresh medium containing no epidermal growth factor for another 48 hr. Four of the six cell cultures were treated with 2.5 or 5 μg/ml bleomycin for 48 hr, with one change of medium at 24 hr to avoid drug depletion. Treated and untreated cultures were allowed to recover for 4 hr in fresh medium before they were subcultured and grown for 8-9 days to express the *HPRT*- phenotype. At the same time, 800 cells were taken from each culture after the treatment of bleomycin and plated in normal MEGM medium for determination of cell survival. At the end of expression period, cells were plated in medium containing 6-thioguanine for *HPRT* - mutant selection. Meanwhile, another aliquot of 800 cells was taken from each culture and plated in normal MEGM medium for determination of plating efficiency. After growth for 8-10 days, the number of 6-thioguanine-resistant mutant colonies in each culture were counted and the mutation frequencies were calculated.

A total of five mutagenesis assays were conducted with 184B5 cells and three with 184B5-E6*tfx*C6 cells. On average, the treatment with 2.5 μg/ml bleomycin increased the mutation frequency by 4- to 5-fold over background in both cell lines, with little or no additional increase at 5 μg/ml. **Table 2** shows the mean and standard error for log(survival) and mutation frequency at each dose for both cell lines.

Northern analysis of *HPRT* mRNA expression was performed on most of the mutants. However, while nearly all mutants showed detectable *HPRT* expression, there were unexpectedly large variations between individual mutants, including many that were later determined to be simple missense base substitutions. For this reason, Northern analysis was not particularly helpful in mutant characterization.

Task 4: Mapping and sequencing of *HPRT* deletions/rearrangements

Task 6: Extension of mapping and sequencing of *HPRT* deletions and rearrangements to c-*myc* and/or other genetically altered strains.

Near the end of year 2, it became apparent that given the resources available, detailed mutation spectra could only be obtained for two different cell lines. Given that no suitable mammary epithelial or breast tumor cell lines differing specifically in c-*myc* expression had been constructed by that time, it was decided to focus the mutation analysis on the 184B5 and 184B5-E6*tfx*C6 cell lines, differing specifically in p53 status. It was also decided that the experiments with the two lines should be done by the same personnel, and concurrently rather than sequentially, to minimize the possibility that differences in mutation spectra might be attributable to subtle differences in culture conditions rather than intrinsic differences between the cell lines.

A total of 52 bleomycin-induced and 33 spontaneous mutants from the 184B5 cell line, and 57 bleomycin-induced and 27 spontaneous mutants from 184B5-E6*tfx*C6 cell line were analyzed. For each mutant, cell RNA was extracted and reverse transcription was performed to synthesize first strand *HPRT* cDNA. The synthesized cDNA was amplified by nested two-stage PCR, using primers suggested by McGregor et al. (1991). PCR products were analyzed by electrophoresis on 1% agarose gels (**Figure 12**). By comparison with the normal-size DNA band (lane 1), deletions could be detected from the DNA band shift, as shown in lane 6 of panel A and lane 5 of panel B.

For each mutant that gave PCR products, nine *HPRT* exons were sequenced using three different primers, with two different gel loading times for each primer in order to visualize the entire region sequenced by each primer. Primer 2 was usually used to sequence exons 3-6 first because mutations tended to occur more frequently in these exons. **Figure 13** shows a typical sequencing gel of a bleomycin-induced mutant with a 7-bp deletion starting at position 141.

The types of spontaneous and induced mutations recovered from each cell line are summarized in **Table 3**. Most of the spontaneous mutations (64% in 184B5 cell line and 59% in 184B5-E6*tfx*C6) were base substitutions, as were about half of the bleomycin-induced mutations in both cell lines. The spontaneous base substitutions comprised approximately equal numbers of transitions and transversions in the 184B5 cell line but four times as many transversions as transitions in the 184B5-E6*tfx*C6 cell line. Among bleomycin-induced base substitutions, the ratio of transitions to transversions was slightly higher (12:10) in the 184B5 cell line, and slightly lower (12:14) in the 184B5-E6*tfx*C6 cell line. **Figure 14** shows the spectrum of spontaneous and bleomycin-induced mutations in *HPRT* cDNA for 184B5 and 184B5-E6*tfx*C6 cell lines. Base substitutions are shown above the nucleotides that had been replaced and the deletions are shown under the nucleotides with lines indicating the extent of the deletion. Bold letters represent bleomycin-induced base substitutions and regular letters represents spontaneous ones, with mutations induced in 184B5-E6*tfx*C6 cells shown in italics. To assess whether bleomycin-induced base substitutions were targeted to potential sites of bleomycin-induced damage (G-$\underline{C}$ and G-$\underline{T}$ sequences) the distribution of mutations among target and nontarget sites was compared to the total incidence of target and nontarget sites in the coding exons. There was no significant correlation between the two, suggesting that the substitutions were untargeted and thus probably arose by some mechanism other than replication or repair of a damaged DNA template, perhaps as a result of a persistent global decrease in replication fidelity (Chang and Little, 1992).

Most bleomycin-induced deletions were small in size, ranging from 1 bp to 8 bp in both cell lines **(Table 4).** Spontaneous deletions were also small, ranging from 2 to 7 bp in both cell lines with one exception of a 49-bp deletion from the 184B5-E6*tfx*C6 cell line. Single base-pair deletions were not observed among spontaneous mutations in either cell line and appeared to be induced specifically by bleomycin. In contrast to the base substitutions, most of the -1 deletions were targeted to potential sites of bleomycin-induced strand breaks **(Figure 14)**. This correlation was statistically significant, with $p<0.0005$ for combined data from both cell lines **(Table 5)**. Moreover, whereas bleomycin can induce DSBs with either blunt ends or single-base 5′ overhangs (depending on sequence), all but one of the targeted -1 deletions occurred at potential blunt-end DSB sites. **Figure 15A** shows a proposed model of how removal of the phosphoglycolate sugar fragment from the 3′ end of the break, followed by blunt-end ligation, would result in deletion of the base pair initially attacked. It is notable that the one -1 deletion at a potential staggered cleavage site occurred at a GTTA•TAAC sequence, at which the predicted one-base 5′ overhangs would be complementary (A•T) and whose annealing prior to ligation would likewise result in a -1 deletion **(Figure 15B)**. *In vitro* end-joining studies suggest that the noncomplementary 5′ overhangs that would be formed at most staggered DSBs would be filled in prior to ligation, with the result that no mutation would occur. Thus the occurrence of -1 deletions at sites of blunt but not staggered DSBs suggest that they arose by end-joining repair of the DSBs rather than by any processing of single-strand breaks or other single-strand lesions. There were also three bleomycin-induced mutants from the 184B5-E6*tfx*C6 cell line, each of which showed deletion of two repeated nucleotides at a bleomycin double-strand break target site at bp 526-527 **(Figure 14).**

In the 184B5 cell line, one of 33 spontaneous and 1 of 52 bleomycin-induced mutations were found to have a normal *HPRT* cDNA sequence for all nine exons, as did two of 57 bleomycin-induced mutants in the 184B5-E6*tfx*C6 cell line. Other investigators have reported analogous cases of 8-azaadenine mutants with no detectable APRT mutation in the CHO-D422 cell line (Povirk et al., 1994; Han et al., 1993; Wang et al., 1994). One possible explanation for these 6-thioguanine selected mutants is that the regulatory sequences outside the coding regions of the *HPRT* gene could be altered and consequently the HPRT enzyme expression level could be very low. In this case, the cells would be able to survive in selective medium containing 6-thioguanine. mRNA from all mutant lines was subjected to Northern analysis with an *HPRT* cDNA probe, and these mutants did tend to show relatively low *HPRT* cDNA content; however, many mutants with missense mutations in the protein coding region also showed low *HPRT* mRNA levels, so the significance of these differences is uncertain. These mutants were not further investigated.

The frequency of mutations resulting in exon skipping was quite high in bleomycin-induced as well as spontaneous mutants isolated from both cell lines, between 20% and 30% in each case. In order to examine the molecular nature of these exon-skipping mutations, genomic DNA was extracted from each of the mutants. The intron / exon junctions were amplified using primers spanning the junction regions for each of the mutants, and the amplified genomic DNA fragments were sequenced. Overall, 48% of bleomycin-induced and 87% of spontaneous exon-skipping mutations from both cell lines showed unambiguous DNA sequence changes. In most cases exon skipping was caused by a single-base substitution in or close to the splice junction sites, with two exceptions of bleomycin-induced mutations where one single-base deletion and one base substitution within the exons resulted in exon skipping **(Figure 16)**. Other researchers also reported a similar high frequency of *HPRT* exon skipping caused by splicing mutations in human T and B lymphocytes (Nelson et al., 1994; Recio et al., 1990).

There were 4 mutants, 2 bleomycin-induced and 2 spontaneous, in which the last 6 bp from 3′ end of exon 2 was deleted. Amplification and sequencing of exon 2 genomic DNA revealed a small range rearrangement in three of the four mutants **(Figure 17)**, which resulted in the deletion of last six bp in exon 2. Other investigators reported a similar deletion of the first 5 bp from the 5′ end of exon 2 due to the creation of a cryptic intron 1 splice acceptor site just inside the exon (Nelson et al., 1994).

Southern analysis was conducted on the several exon-skipping mutants that did not show changes in splice junction DNA sequences. Interpretation the banding pattern changes was complicated by the existence of two copies of *HPRT* gene, and although several of these mutants showed bands of reduced relative intensity consistent with partial allele loss, only two showed unambiguous changes in banding pattern. As shown in **Figure 18**, mutant E6C6IIA$_2$C$_1$ had an additional DNA fragment of approximately 9.1 kb, and mutant E6C6IIB$_3$I$_4$ showed a new fragment of approximately 6.5 kb when compared with their parental cell line 184B5-E6*tfx*C6; both these mutants also clearly had reduced relative intensity of the 7.8-kb band. Thus, these mutants had a deletion or rearrangement within *HPRT*, estimated to be on the order of several hundred base pairs based on the changes in fragment size.

Initially, one goal of the proposal was to determine whether cells sustaining a bleomycin-induced chromosomal rearrangements involving the *HPRT* gene would have also acquired a global loss of chromosomal stability, as indicated by the presence of multiple chromosomal re arrangements and changes in those rearrangements over time. If so, we would then proceed to examine other endpoints of genomic instability such as delayed reproductive death, loss of competence for apoptosis and lack of DNA damage-induced cell cycle arrest. However, the lack of any detectable interchromosomal rearrangements involving the *HPRT* locus precluded such analysis. Nevertheless, DNA radiation-induced cell cycle perturbations were examined in several bleomycin-induced *HPRT* mutants of 184B5 cells, and in all cases the intact G$_1$ block seen in the parental line was retained in the mutants (data not shown). Cytogenetic studies were also carried out on a limited number of mutants in the laboratory of Dr. Colleen Jackson-Cook in the Department of Human Genetics. The results showed a set of consistent chromosome aberrations, previously reported by Walen and Stampfer (1989), that were carried in parental cell line as well as derived mutant lines **(Table 6)**. In addition, some incidental cytogenetic findings were identified in some of the mutant lines **(Table 7)**. These incidental chromosome changes could be generated from misrepair of DNA DSBs induced by bleomycin. The abundance of marker chromosomes might suggest genetic instability in some of these mutant lines. In addition, spectral karyotyping (SKY), in which each human chromosome is distinguished by fluorescent labeling, was performed for a few selected mutants. The spontaneous mutant line A_1F_5 had an exon 5 skipping mutation at DNA sequence level. SKY analysis showed that this cell line carried the basic set of chromosome aberrations that was carried through in parental cell line 184B5 **(Figure 19)**. The bleomycin-induced mutant line B_21F_4 had an rearrangement involving 164 bp at DNA sequence level. SKY analysis showed that other than the basic set of chromosome aberrations observed in parental cell line 184B5, it also carried a chromosome 1 derivative containing part of chromosome 15, a chromosome 5 derivative containing part of chromosome 1, and other aberrations **(Figure 20).** A limited number of spontaneous and bleomycin-induced mutants are currently being analyzed to determine whether such additional chromosomal changes are a consistent feature of bleomycin-induced mutants.

The absence of any mutations involving interchromosomal translocations raised the question of whether translocations were not induced under the conditions used, or whether translocation-containing cells were lost as the cells proliferated. To address this question 184B5 cells were harvested either 2days or 30 days after bleomycin treatment and subjected to SKY. Results of this experiment showed that numerous translocations, in addition to the constitutive translocations in the parent line, were present at day 2, but were much reduced by day 30 (**Table 8**). However, no translocations involving the X chromosome were detected at any time. Thus, the lack of mutants involving translocations may have been due to two factors: a relative immunity of the X chromosome from translocation formation , as reported previously for irradiated cells (Jordan and Schwartz, 1994), and a further selection against at least some translocation-containing cells during proliferation.

In summary, bleomycin-induced mutations at the *HPRT* locus in mammary epithelial cells were predominantly point mutations, the majority of which were base substitutions, with no significant qualitative or quantitative differences between $p53^+$ and p53- sublines. Bleomycin-induced deletions were small, rarely more than a few base pairs. Bleomycin-induced single-base deletions were clearly targeted to sites of double-strand breaks and probably arose errors in repair of such breaks. However, base substitutions were apparently untargeted, and so probably arose from an induced reduction in replication fidelity. Only a few rearrangements were detected, none of which appeared to extend beyond the *HPRT* locus. Large deletions and rearrangements, which typically dominate spectra of mutations induced by ionizing radiation in mammalian cells, were conspicuously absent. Likewise, there were apparently no bleomycin-induced interchromosomal translocations, such as those seen at the *aprt* locus in Chinese hamster cells following treatment with bleomycin under identical conditions (Povirk et al., 1994).

KEY RESEARCH ACCOMPLISHMENTS

- Substantiation of the absence of apoptotic cell death in breast tumor cells exposed to radiation or adriamycin.

- Establishment of the concept that Vitamin D3 analogs can be utilized to promote apoptotic cell death in the breast tumor cells at least in part, through sensitization of the cell to apoptotic cell death.

- Development of a new model for enhancement of exogenous gene delivery and expression in breast tumor cells (utilizing estradiol or irradiation).

- Identification of a senescence response to adriamycin and ionizing radiation that may be linked to p53 and c-myc.

- Demonstration of the loss of G1/S checkpoint in the 184B5-E6*tfx*C6 derivative mammary epithelial cell line.

- Determination of the spectrum of spontaneous mutations in 184B5 ($p53^{+}$) and 184B5-E6*tfx*C6 (p53-) mammary epithelial cells, the first such spectrum in any cells of breast tissue origin.

- Detection of errors in double-strand break repair, as targeted single-base deletions in bleomycin-treated $p53^{+}$ and p53- mammary epithelial cells.

- Analysis of cytogenetic damage in bleomycin-treated mammary epithelial cells by 24-color *in situ* fluorescence labeling, demonstrating selection against chromosomal translocations as the cells proliferate.

- Molecular analysis of over 100 bleomycin-induced *HPRT* mutants in $p53^{+}$ and p53- mammary epithelial cells, demonstrating conspicuous lack of large-scale deletions and rearrangements; this is the first such analysis for any DNA-damaging agent in cells of breast origin, the first for any radiomimetic drug in any human cell system and one of only a few studies in any cells of female origin.

REPORTABLE OUTCOMES

- Manuscripts

Wang Z. Gewirtz, DA, Grant S. Modulation of leukemic cell radiosensitivity by enforced expression of p53 or dysregulation of $p21^{CIP1}$. In preparation.

Di YM, Akalin A, Holt SE and Gewirtz DA. Suppression of telomerase with induction of senescence in p53 wild-type MCF-7 breast tumor cells and delayed apoptosis in p53-mutated MDA-MB231 breast tumor cells after acute exposure to adriamycin. Submitted.

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- Presentations

Jones KR and Gewirtz DA. Influence of adriamycin on growth arrest and cell death pathways in the p53 mutated T-47D breast tumor cell line. Presented at the Annual Meeting of the American Association for Cancer Research, 2000.

Magnet KJ, Orr MS, Cleveland JL and Gewirtz DA. C-myc in the DNA damage response pathway. Presented at the Annual Meeting of the American Association for Cancer Research, 2000.

Gewirtz DA, Di YM, Randolph JK and Jain PT. Suppression of E2F activity associated with growth arrest in human breast tumor cells exposed to a pharmacological concentration of estradiol. Presented at the Annual Meeting of the American Association for Cancer Research, 2000.

Chaudhry M, Sundaram S, Reardon DB and Gewirtz DA. The Vitamin D analog ILX23-7553 enhances the response to adriamycin and radiation in breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, 2000.

Di YM, Bronder J and Gewirtz DA. Relationship of p53 status to adriamycin sensitivity and resistance to apoptosis in MCF-7 and MDA-MB231 breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, 1999.

Sundaram S and Gewirtz DA. EB 1089 radiosensitizes breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, 1999.

Magnet KJ, Sundaram S and Gewirtz DA. Analysis of E2F function in response to DNA damaging agents in breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, 1999.

Chen, S. and Povirk, L.F.: Processing of terminally blocked DNA double-strand break ends *in vitro* and *in vivo*, and the role of DNA-PK. Invited presentation, 6th Intl. Workshop on Radiation Damage to DNA, Chapel Hill NC, April 17-21, 1999.

Yu, Y., and Povirk, L.F.: Genomic instability and gene rearrangements induced by radiomimetic antibiotic bleomycin in nontransformed 184B5 mammary epithelial cells. Presented at the annual meeting of the Environmental Mutagen Society, Mar. 27 - Apr 1, 1999, Washington D.C.

Magnet KJ and Gewirtz DA. Influence of ionizing radiation on proliferation and c-myc expression in twp p53 positive breast tumor cell lines. Presented at the Annual Meeting of the American Association for Cancer Research, March 28 - April 1, 1998, Philadelphia, PA.

Sundaram S and Gewirtz, D.A. EB 1089 enhances the antiproliferative effects of radiation in breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, March 28 - April 1, 1998, Philadelphia, PA.

- Funding applied for based on work supported by this award

We have recently submitted the following research proposals:

Department of Defense Breast Cancer Research Program
Clinical Translational Award
Utilization of Vitamin D analogs to enhance the response of breast cancer to chemotherapy

Department of Defense Breast Cancer Research Program
IDEA Award
Reciprocal Regulation of apoptosis and senescence by adriamycin in the breast tumor cell

National Institutes of Health
Vitamin D3 analogs and adriamycin in breast cancer
Support Requested for 4 years at ~ $200,000/year

We have obtained the following additional support based on the funding applied by this award.

American Institute for Cancer Research
July 1, 1999- June 30, 2000
Enhancement of the response to ionizing radiation in the breast tumor cell by Vitamin D3 analogs
Total Direct Costs, Approximately $150,000

American Institute for Cancer Research
January 31, 2000 - January 30, 2002
Postdoctoral Fellowship for Dr. Mona Gupta
Total Direct Costs ~ $50,000

ILEX Products
June 1, 1999- May 30, 2000
Utilization of the Vitamin D3 analog ILX23-7553 to enhance the response to ionizing radiation and adriamycin in the breast tumor cell.
Total Direct Costs, $20,000

- Degrees granted

Mahreen Chaudhry, M.S. Degree granted May 2000
Yin Yu, Ph.D. degree expected, November 2000

-Patents applied for:

1. EB 1089 enhances the efficacy of fractionated radiation therapy in breast tumor cells. Gewirtz and Gupta.

2. Combination of the Vitamin D3 analog ILX23-7553 with adriamycin or irradiation against breast tumor cells. Chaudhry and Gewirtz.

3. Combination of the Vitamin D3 analog EB 1089 with adriamycin against breast tumor cells. Sundaram and Gewirtz.

Personnel Receiving Pay from this research Effort

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CONCLUSIONS

Conclusions Related to Technical Objectives 1 through 4

- One of our primary conclusions is that breast tumor cells are refractive to chemotherapy and radiotherapy induced apoptosis. The implication of this finding is that the recurrence of disease could be a consequence of the absence of apoptotic cell death in metastatic breast cancer. Consequently, we have modified our goals to develop approaches to enhance the sensitivity of the breast tumor cell to radiation and drugs such as adriamycin.

- We have developed two primary approaches which we ultimately hope to test both in an animal model system and, if successful, in the clinical setting. One approach is to combine radiation or adriamycin with Vitamin D analogs which are not hypercalcemic. It is anticipated that these combinations could lead to more effective cell killing at conventional (or reduced) doses of drugs and radiation.

- We have also developed approaches for enhancing the uptake and expression of exogenous genes (using either high dose estradiol or radiation). These studies may ultimately have utility in the area of gene therapy to increase the delivery of genes which promote cell death. .

- We have recently determined that both adriamycin and ionizing radiation induce replicative senescence in the p53 wild-type MCF-7 breast tumor cell and delayed apoptosis in the p53 mutant MDA-MB231 breast tumor cell. Taken together with our earlier findings that adriamycin and radiation suppress expression of c-myc which is predictive of growth arrest, these observations suggest a linkage between p53, regulation of c-myc expression (and function) and the likelihood that the cell will arrest rather than undergoing apoptosis. The propensity of the cell to respond through growth arrest rather than (apoptotic) cell death may allow for repopulation, a phenomenon associated with the response to fractionated radiation(Schmidt-Ullrich et al, 1999) and recovery of proliferative capacity. Further analysis of this response pathway may provide insights into the basis for disease recurrence subsequent to both radiotherapy and chemotherapy.

Conclusions Related to Technical Objectives 5 and 6.

- Of the mutations induced by bleomycin in mammary epithelial cells, only the single-base deletions appear to be targeted to sites of bleomycin damage. The fact that these deletions nearly always occur at potential sites of bleomycin-induced blunt-ended double-strand breaks (but rarely at sites of staggered DSBs, which are nearly as frequent), suggest that they arose by direct end-joining of those breaks, with deletion of the base pair originally destroyed in formation of the break (**Figure 15**). These events suggest that a significant number of double-strand breaks were in fact induced by bleomycin in the target gene, and were repaired by an end-joining pathway.

- What was surprising, however, was the complete lack of bleomycin-induced large-scale deletions and rearrangements. Although there have been only a few studies of bleomycin-induced mutagenesis in mammalian cells, extensive work with ionizing radiation has suggested that free radical-mediated double-strand breaks (the primary lesion common to both agents) result mainly in large-scale deletions and rearrangements, presumably as a result of misrepair of the breaks. The predominance of point mutations among the *HPRT* mutants induced by treatment of mammary epithelial cells with bleomycin was thus unexpected, and clearly different from *HPRT* spectra generated in irradiated cells under a variety of conditions. Any of four factors (or a combination thereof) could account for the lack of induced deletions/rearrangements in the present study: (1) intrinsic differences between bleomycin and radiation in the nature of the initial DNA damage, (2) the nature of the *HPRT* locus in mammary epithelial cells, (3) the fact that the cells were treated in plateau phase (most previous studies having been done in exponentially growing cells), or (4) a particularly strong suppression of or selection against translocations the mammary epithelial cells.

● With regard to the first possibility, one difference between bleomycin and ionizing radiation is that the latter often induces DSBs in clusters at a single ionization track. Such clustered breaks would be more likely to result in misjoining of exchanged ends. This question could be addressed by analysis of mutations induced in the same cells by irradiation in plateau phase.

● With regard to the second possibility, nearly all molecular characterization of *HPRT* mutants has been performed with cells of male origin, precisely because the lack of an inactive homologue makes such characterization much easier. It is possible that in mammary epithelial cells, the presence of an intact homologous copy of *HPRT* protects against large deletions and rearrangements being generated during DSB repair, due to homologous recombination between the two alleles or other homology-dependent mechanisms. Examining bleomycin-induced mutagenesis at the *HPRT* locus in plateau-phase cells of male origin, or at some other locus that is physically as well as functionally hemizygous, could serve to address this question.

● With regard to the third possibility, it is notable that large deletions constituted a substantial fraction of *hprt* mutants induced by bleomycin in exponentially growing V79 hamster lung fibroblasts (Köberle and Speit, 1991), but were entirely absent among *aprt* mutants induced by bleomycin in plateau-phase CHO-D422 cells (Povirk et al., 1994). While this difference could be due to the much smaller size of the *aprt* gene, it could also be due to replication-dependent deletion mechanisms, for example collision of replication forks with DSBs, which would only contribute to mutagenesis in exponentially growing cells.

● With regard to the fourth possibility, it is notable that although bleomycin treatment of plateau-phase CHO-D422 cells did not result in any large deletions, it did produce several interchromosomal reciprocal translocations, apparently resulting from aberrant joining of the exchanged ends of two double-strand breaks on different chromosomes (Wang et al., 1997). By comparison, the lack of bleomycin-induced translocations in the present study is especially striking considering the difference in target sizes for the two types of mutations. In order to produce a cell with a mutant phenotype, a single-base deletion or other point mutation must occur within the exons or splice junctions of a gene, while a translocation could occur anywhere in the gene locus. Since the *HPRT* locus (40 kb) is ~20 times larger than the *aprt* locus (2.1 kb), while the protein-coding and splice-junction sequences for the two genes are comparable

(~700bp), the relative frequency of translocation mutants should be much higher at the *HPRT* locus, assuming that the fraction of DSB ends that undergo exchange during rejoining is the same. Thus, the fact that single-base deletions but no translocations were detected among bleomycin-induced *HPRT* mutants in either 184B5 or 184B5-E6*tfx*C6 cells suggests that the intrinsic frequency of viable translocations was much lower (by at least tenfold) in these cells than in CHO-D422 cells. This difference could result from either a lower initial incidence of translocations in the mammary cells (perhaps because translocations are somehow suppressed due to the presence of a homologous copy of the locus), or from a selection against translocations after they occur. Previous work has shown that, following irradiation of human lymphocytes, the fraction of cells containing reciprocal translocations slowly decreases as the cells proliferate, suggesting that there was some sort of selection against translocations, even (theoretically viable) balanced reciprocal translocations (Hoffmann et al., 1999). SKY studies (**Table 8**) suggest that a similar selection against translocations may occur in mammary epithelial cells. Despite the apparent tolerance for the translocations already present in the parent line, newly acquired translocations seemed to be less well tolerated and were gradually lost from the cell population.

- It may finally be noted that, in all the karyotypes of 184B5 and 184B5-E6*tfx*C6 cells thus far examined (~50 in total) not a single example of a translocation involving the X chromosome has been seen. Thus, the X chromosome may be particularly stable in these cell lines, and less prone to being involved in misrejoining events, as previously reported for human lymphocytes (Jordan and Schwartz, 1994).

- In summary, the results suggest that the mammary epithelial cell genome is remarkably stable in the face of small numbers of DSBs, most of which are repaired either correctly or with loss of only one or a few base pairs. In the small fraction of cases that incorrect ends are joined, most of the resulting translocations are lost as the cells proliferate, due to negative selective pressures that are at least partly p53-independent.

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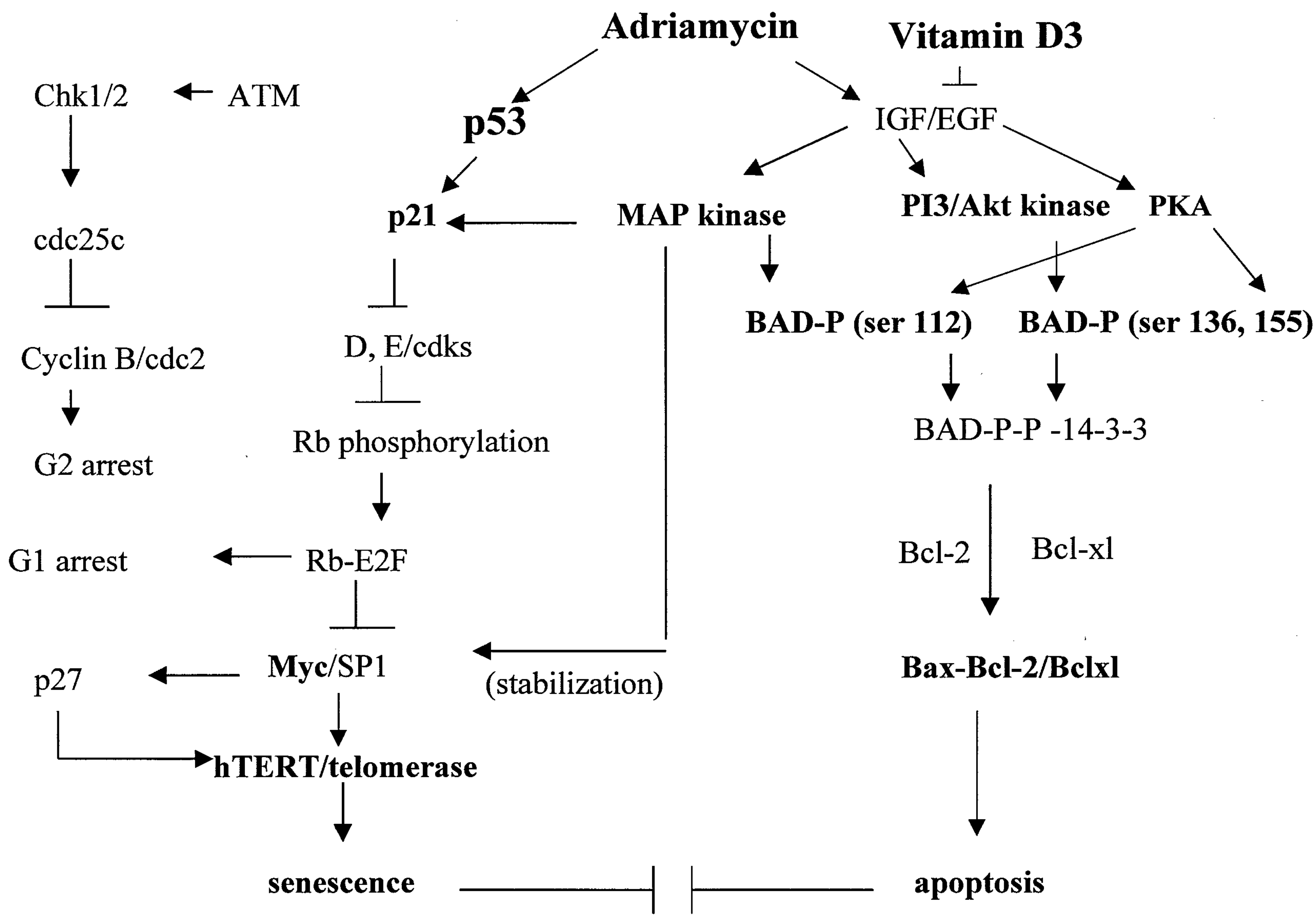
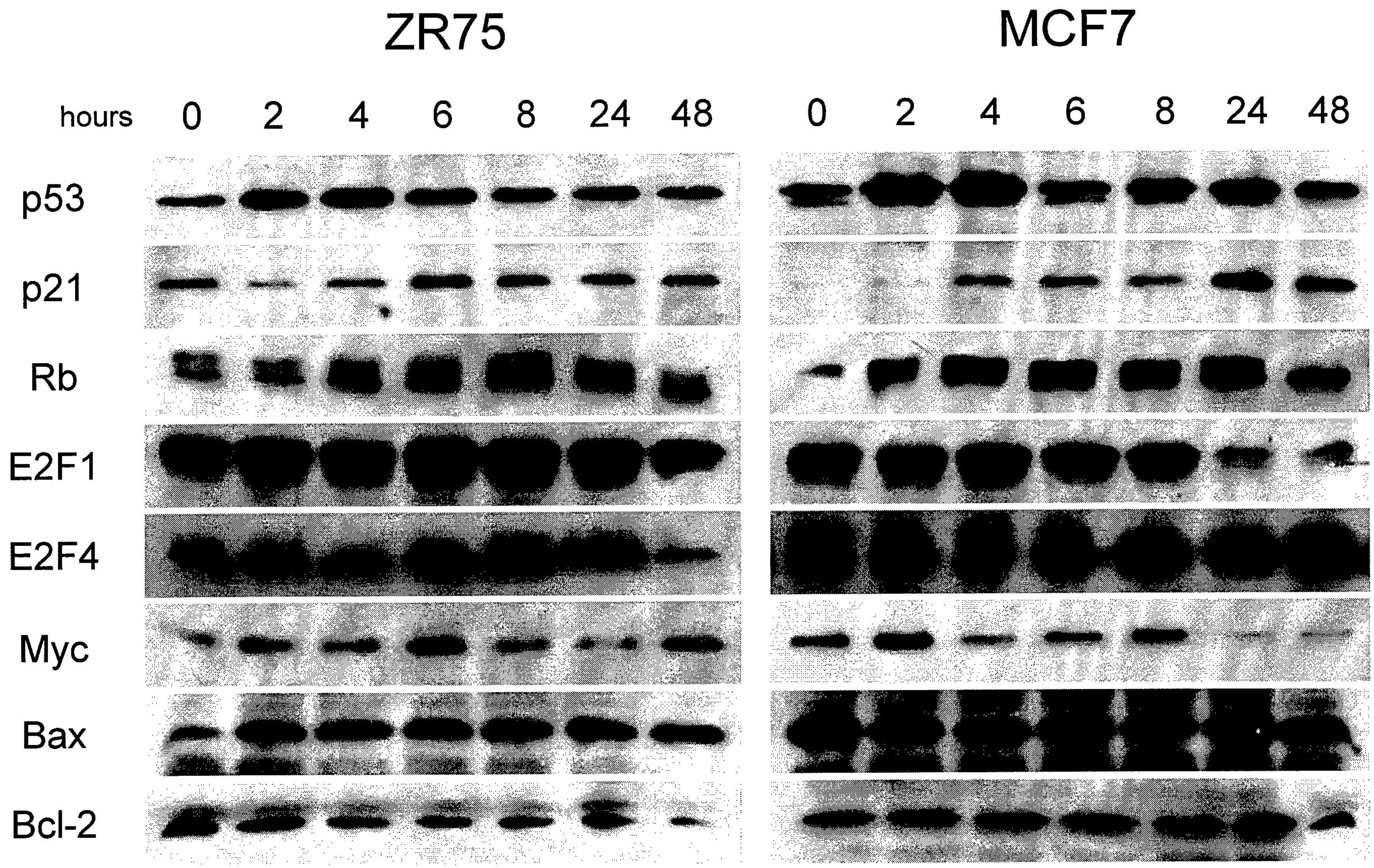


Figure 1. Elements of cell cycle regulation, senescence and apoptosis.

Figure 2. Influence of ionizing radiation on levels of select cell-cycle and apoptosis regulatory proteins. Cells were exposed to 10 Gy of ionizing radiation , protein was extracted at the indicated times, electrophoresed and blotted with the appropriate antibodies.



Mcf-7 transfection with p21AS

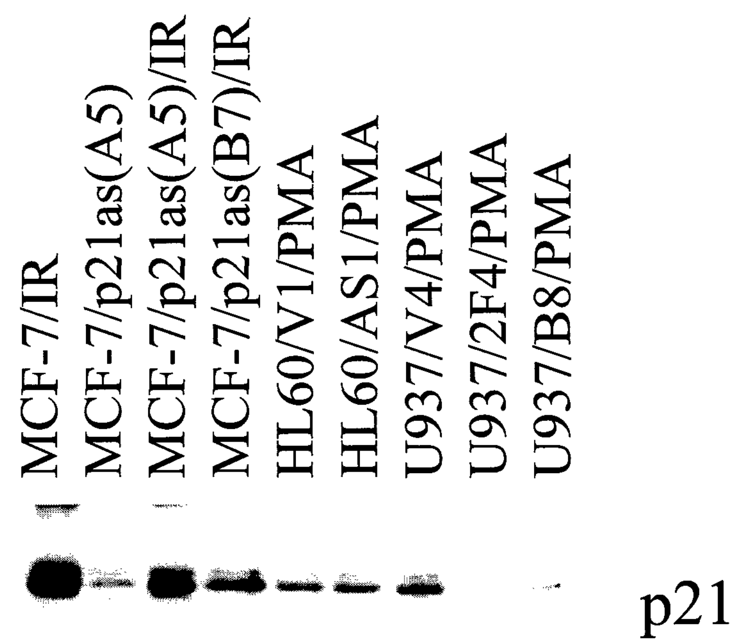


Figure 3.

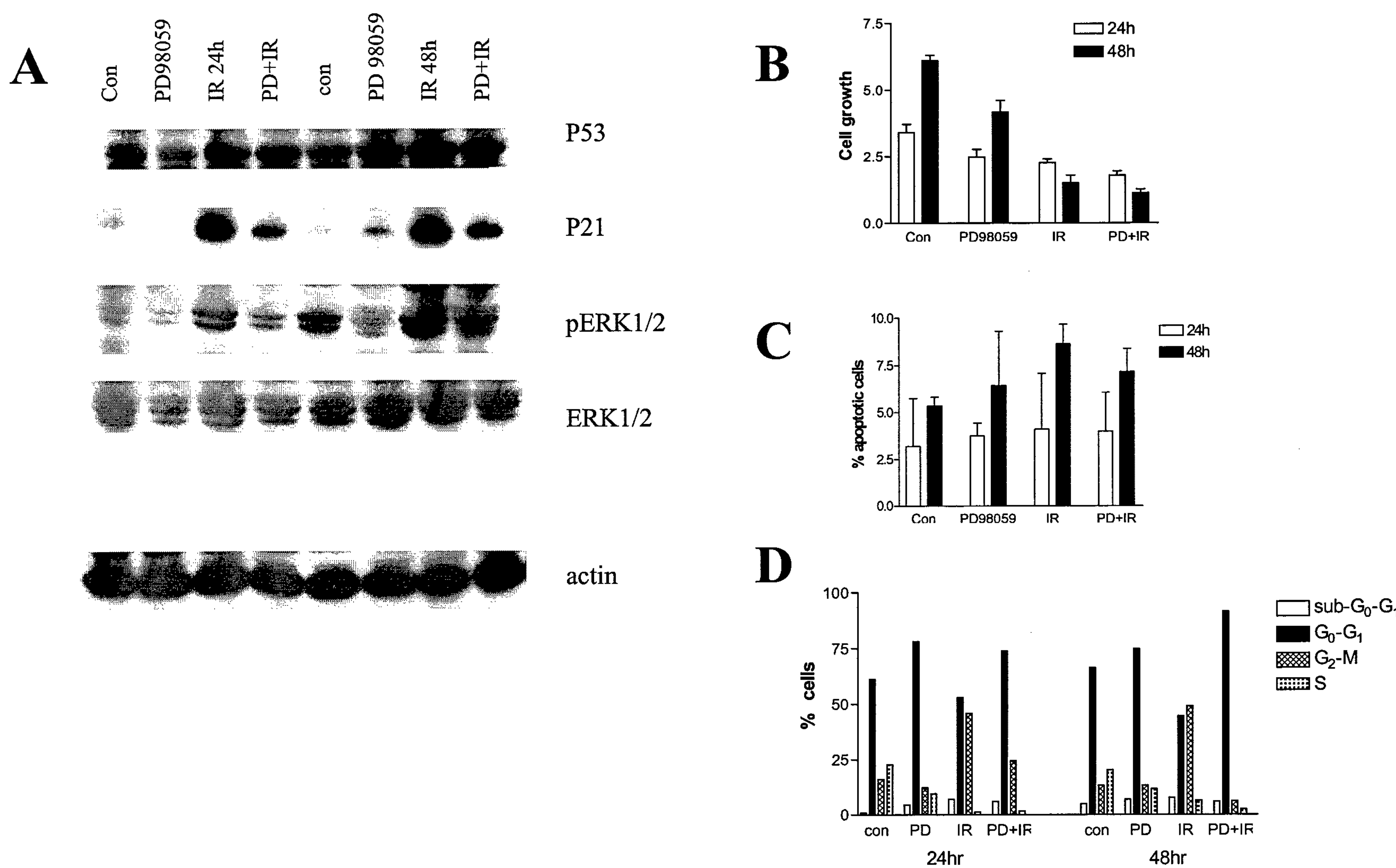


Figure 4. Influence of MAP kinase inhibition on growth arrest and susceptibility to apoptotic cell death in MCF-7 breast tumor cells. Cells were treated with the MAP kinase inhibitor PD98059 and then exposed to 10 Gy of irradiation followed by assessment of p53 and p21 induction (A), cell growth (B), apoptotic cell death (C) and cell cycle distribution (D).

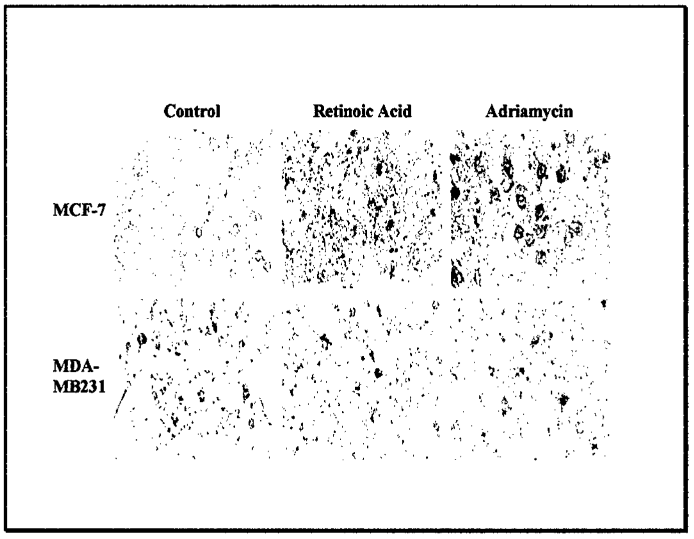


Figure 5: Replicative senescence was identified based on expression of β-galactosidase. A 3-day exposure to retinoic acid (RA) was included as a positive control.

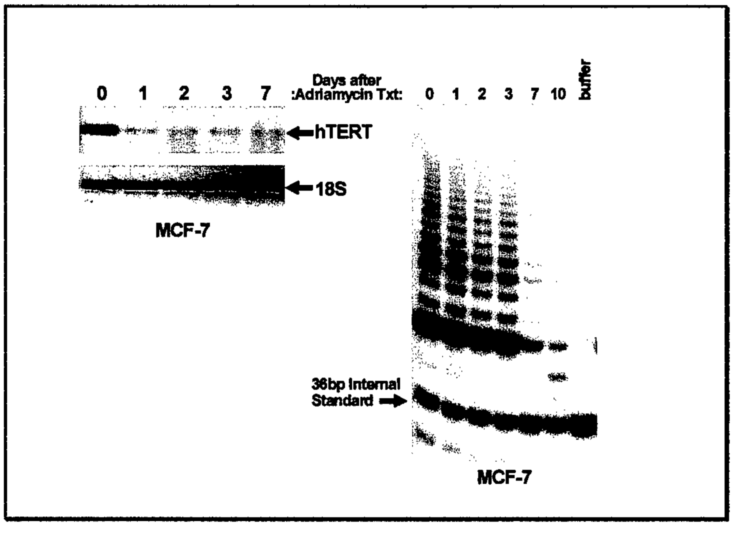


Figure 6. Suppression of hTERT (RT-PCR, left) and of telomerase activity (TRAP assay, right) by adriamycin in MCF-7 cells.

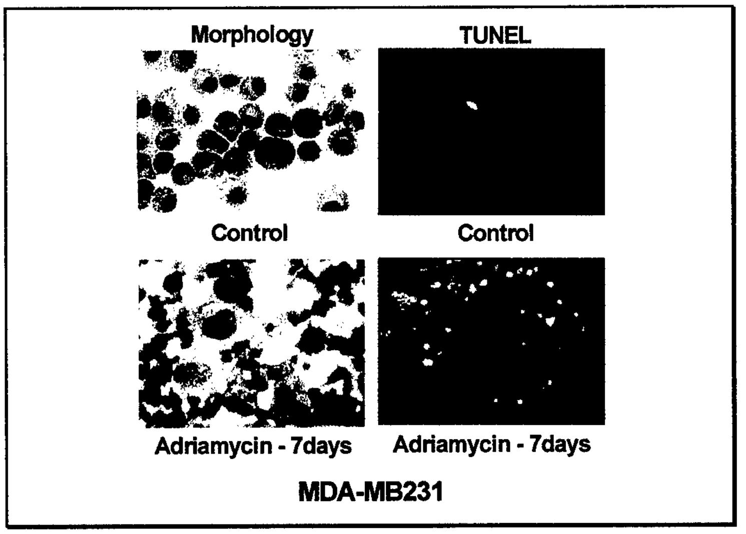


Figure 7 : Delayed apoptosis in response to adriamycin in MDA-MB231 cells. Both morphology and TUNEL were performed on untreated cells (Control) and after acute treatment of MDA-MB231 cells with adriamycin

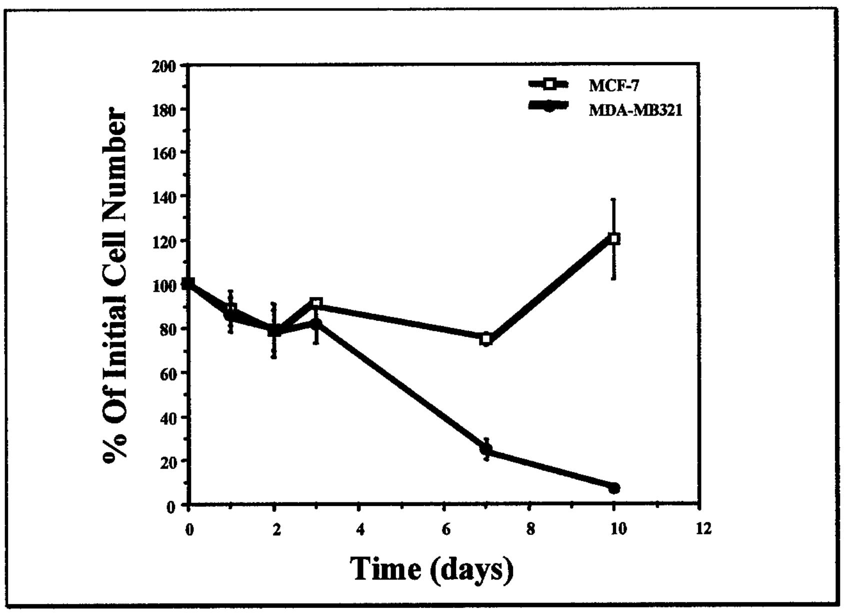


Figure 8. Recovery of MCF-7 cell proliferation after prolonged growth arrest. MCF-7 cells were acutely exposed to 1μM adriamycin, and cell number was monitored for the indicated time. Each point represents the average cell number for duplicate samples.

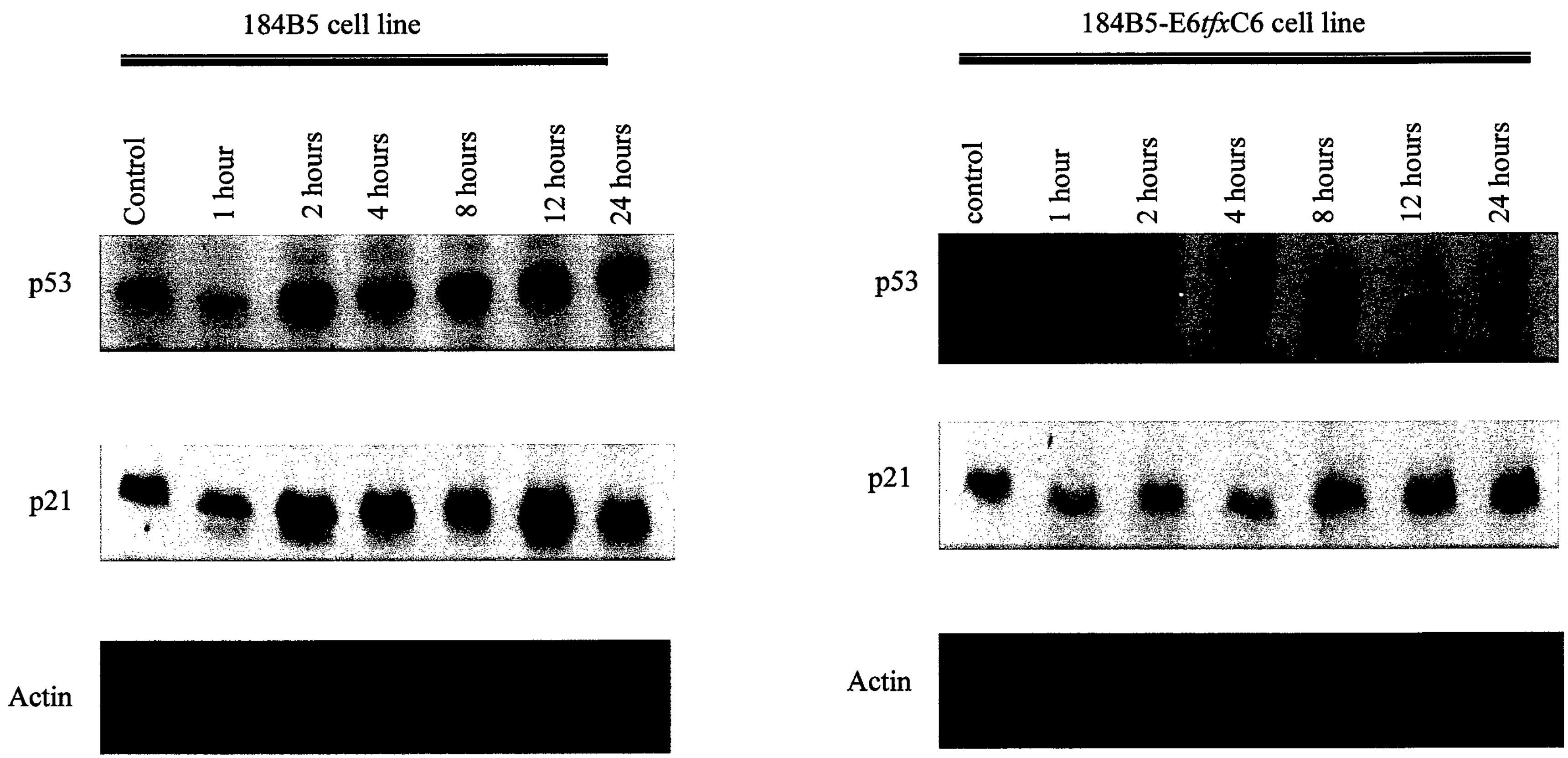


Figure 9: X-ray induced p53 and p21 protein expression in 184B5 and 184B5 E6tfxC6 cell line. Cells were irradiated with 6 Gy X-rays and cultured for various time periods before total cell protein was extracted. In 184B5 cell line, p53 protein expression level is increased greatly 2 hours after the irradiation and starts to go down at 4 hours but the level still remains higher than control 24 hours after irradiation. p21 protein is also induced correspondingly 2 hours after the irradiation, reaches a peak level at 12 hours and remains high 24 hours after irradiation. In contrast, p53 protein has a undetectable basal level in 184B5 E6tfxC6 cell line and is not induced any time after 6 Gy X-ray irradiation. p21 also has a very low basal level and is not induced by x-ray irradiation. Actin was probed on the same blot of p53 protein to verify the equal loading.

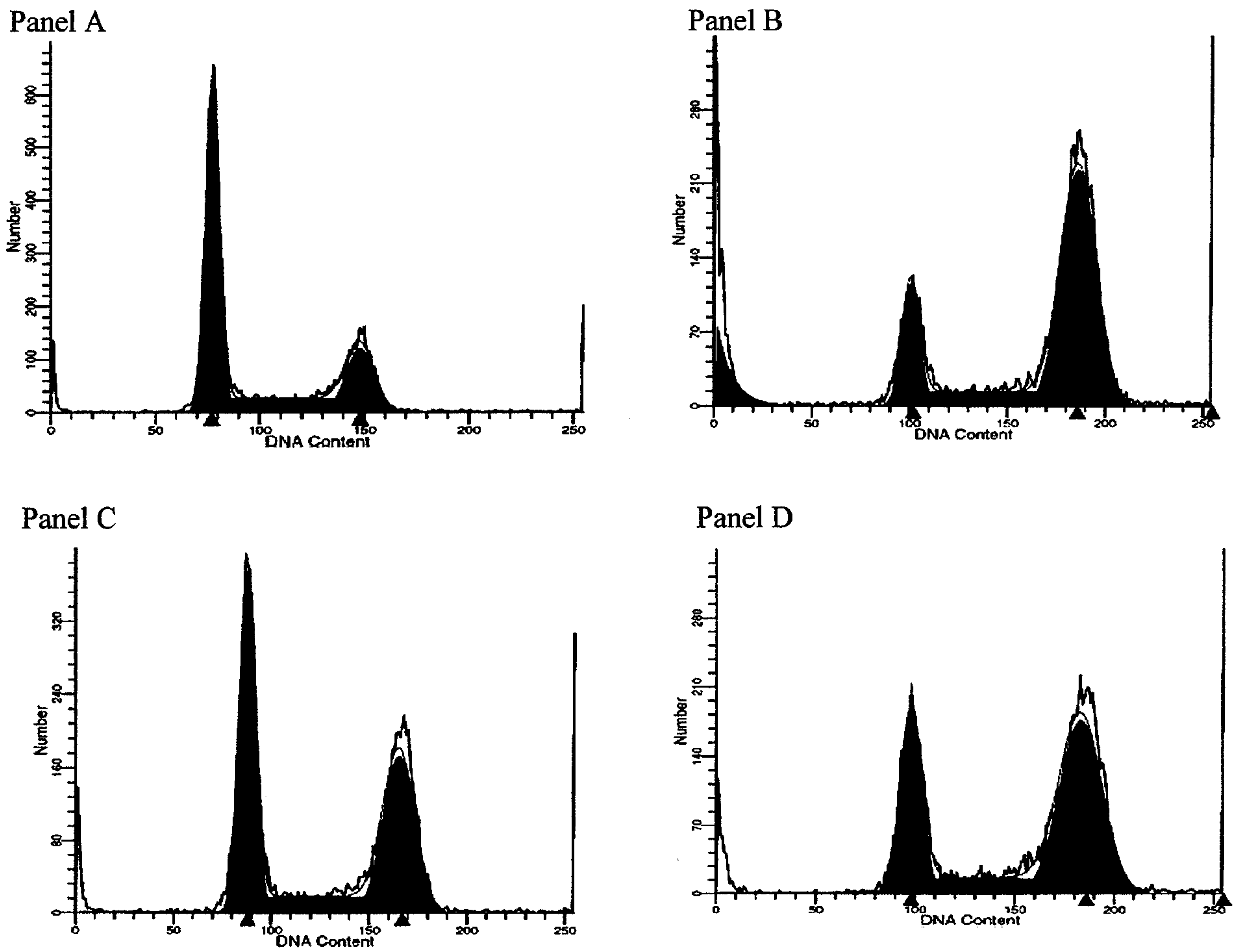


Figure 10: X-ray induced p53-mediated G0/G1 cell cycle checkpoint in the 184B5 cell line. (A) shows the normal cell cycle distribution. (B) shows the cell cycle distribution after nocodazole was added to the cell culture. The majority of the cells are blocked in mitosis by nocodazole. (C) shows the cell cycle distribution after cells were exposed to 6 Gy γ-ray. A large fraction of cells are blocked in G2/M phase upon X-irradiation. (D) shows the cell cycle distribution when nocodazole was added to the culture medium after the cells were irradiated with 6 Gy γ-ray. Under this condition, the cell cycle is shifted to G1 phase in comparison to the condition when nocodazole used alone, indicating an intact G1 checkpoint.

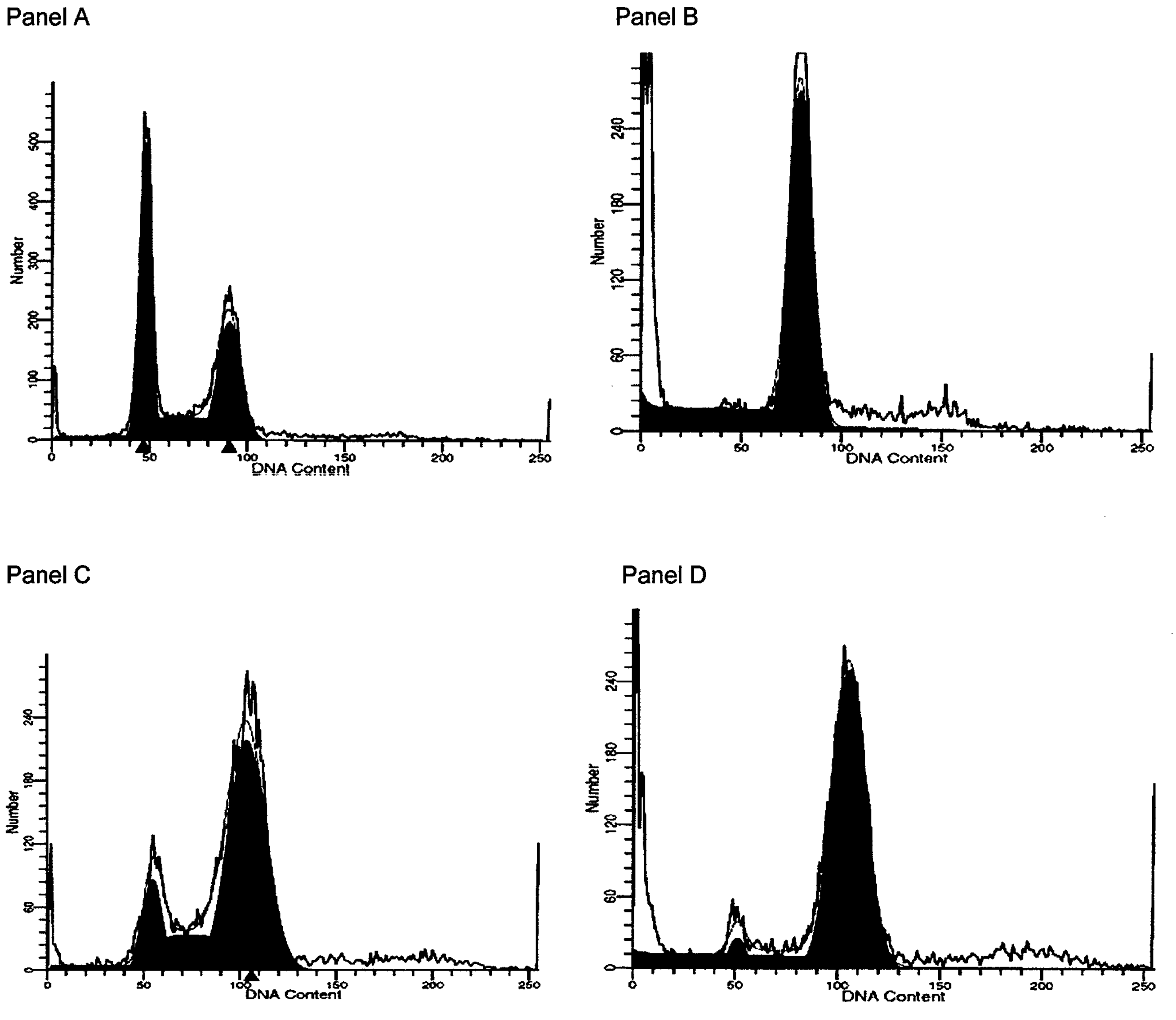


Figure 11: Radiation- induced p53-mediated G_0/G_1 cell cycle checkpoint in 184B5-E6*tfx*C6 cell line. (A) Shows the normal cell cycle distribution. (B) Shows the cell cycle distribution after 50 ng/ml nocodazole was added to the cell culture. Almost all the cells are blocked in mitosis by nocodazole, there are no cells in S phase. (C) Shows the cell cycle distribution after cells were exposed to 6 Gy γ-ray. Most cells are blocked in G_2/M phase upon X-ray irradiation. (D) Shows the cell cycle distribution when nocodazole was added to the culture medium after the cells were irradiated with 6 Gy γ-ray. There is a very small percent of cells are in G_0 phase in comparison to treatment with nocodazole.

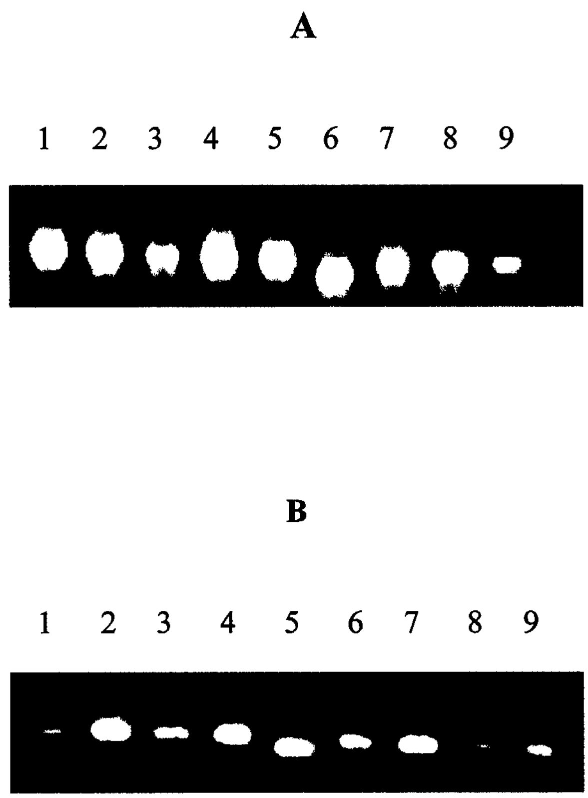


Figure 12: Agarose gel analysis of RT-PCR products. RT-PCR products of HPRT⁻ mutants were analyzed on a 1% agarose gel. (A) shows the PCR products of nine HPRT⁻ mutants from 184B5 cell line. Lane 6 shows that the mutant carried a deletion which was determined later by DNA sequencing as exon 6 deletion. (B) shows the PCR products of nine HPRT⁻ mutants from 184B5 E6*tfx*C6 cell line. Lane 5 shows that the mutants had a small size deletion which was later proved by DNA sequencing as exon 8 skipping.

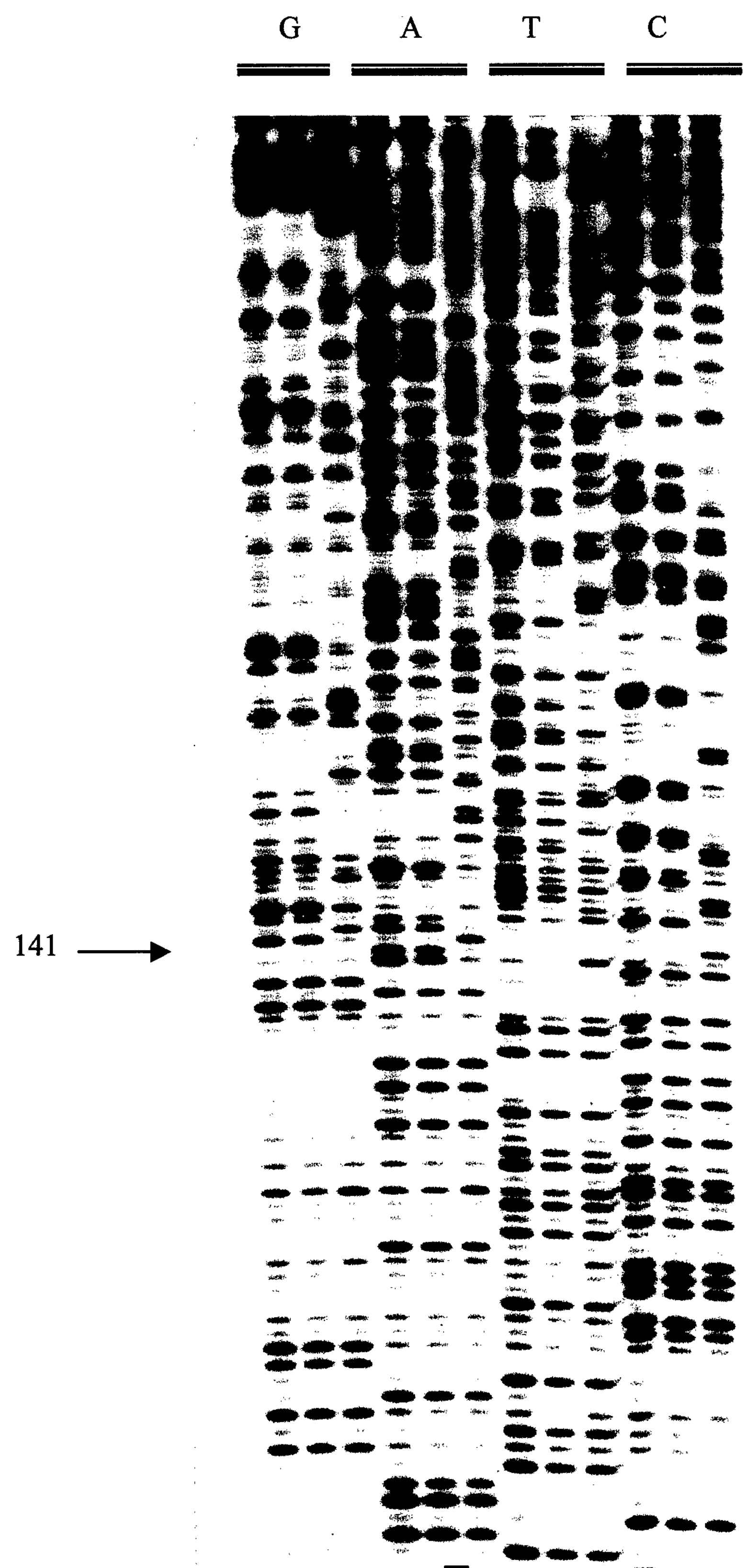


Fig 13: DNA sequencing of mutant HPRT genes. The DNA sequences of exon 1 to 3 from three bleomycin-induced mutants are shown here. Lanes 1 through 3 represent the three mutants analyzed. Letters G, A, T and C indicate the nucleotide the bands represent. Mutant number 3 had a small deletion starting from position 141 and ending at 147. The DNA sequence after the deletion site is shifted down by 7 base pairs in comparison with the other two normal sequences.

```
                        A                            T(2)  T          G   A
1       ATGGCGACCC  GCAGCCCTGG  CGTCGTGATT  AGTGATGATG  AACCAGGTTA  TGACCTTGAT  TTATTTTGCA
                                 -1(3)         -1(2)    -1           -1
                                ____
                                -2

                                                                                T
                        A                                       A               G   C   CA(2)
71      TACCTAATCA  TTATGCTGAG  GATTTGGAAA  GGGTGTTTAT  TCCTCATGGA  CTAATTATGG  ACAGGACTGA
                __________                                                 ________
                  -7(3)   -1                                                 -6(2)
                  -7(2)                                                      -6(2)

                                                        G                            C
141     ACGTCTTGCT  CGAGATGTGA  TGAAGGAGAT  GGGAGGCCAT  CACATTGTAG  CCCTCTGTGT  GCTCAAGGGG
         -7(4)                                      -1
       __________                     _____________________________________________________
            -9                                         -49

                     G(5)                   C
                     G(2)                   C(2)                          A
211     GGCTATAAAT  TCTTTGCTGA  CCTGCTGGAT  TACATCAAAG  CACTGAATAG  AAATAGTGAT  AGATCCATTC
      _____

        G  A                A            T
281     CTATGACTGT  AGATTTTATC  AGACTGAAGA  GCTATTGTAA  TGACCAGTCA  ACAGGGGACA  TAAAAGTAAT

                                                                A(2)
                                                                AA(7)
                                                                C
                                                                CA(4)
             C             G                     T        T     AA(7) C
351     TGGTGGAGAT  GATCTCTCAA  CTTTAACTGG  AAAGAATGTC  TTGATTGTGG  AAGATATAAT  TGACACTGGC
```

Figure 14 (legend on next page)

```
                                        G                      T   G     T
421   AAAACAATGC   AGACTTTGCT   TTCCTTGGTC   AGGCAGTATA   ATCCAAAGAT   GGTCAAGGTC   CAAGCTTGC
                                              -1
                                              -1

                                                                   A
                    G                                              A(2)
                    G                                              C       A                 T(2)
491   TGGTGAAAAG   GACCCCACGA   AGTGTTGGAT   ATAAGCCAGA   CTTTGTTGGA   TTTGAAATTC   CAGACAAGTT
                   -1                         -1  -2(3)
                                                 -1

                            C(2)                                C                            T
              C             C(2)      T                        C(2)                         CT
561   TGTTGTAGGA   TATGCCCTTG   ACTATAATGA   ATACTTCAGG   GATTTGAATC   ATGTTTGTGT   CATTAGTGAA
                                                                                  -7

                          G(2)
631   ACTGGAAAAG   CAAAATACAA   AGCCTAA
```

Figure 14 (begins on previous page): **Spectrum of bleomycin-induced and spontaneous mutations in the HPRT cDNA in the 184B5 and the 184B5-E6*tfx*C6 cell lines.** The coding strand of the nine exons of HPRT cDNA is shown, with primary sites of bleomycin-induced cleavage (G**C** and G**T**) shown in bold (note that **A**'s in the sequence **A**C and **G**'s in the sequence **G**C are primary cleavage sites due to the G**T** or G**C** in the complementary strand). Base substitutions are shown above the nucleotides with which they replaced. Deletions are shown by underlining deleted sequence and the numbers below the line indicate the size of each deletion. The numbers inside the parentheses indicate the number of mutants that have the same sequence change. Bold characters represent the bleomycin-induced sequence changes and regular characters represent spontaneous changes in the 184B5 cell line. Bold italic characters represent bleomycin-induced sequence changes and regular italic characters represent spontaneous changes in the 184B5-E6*tfx*C6 cell line. The adjacent bases at positions 399-400 each represent separate mutations, not tandem mutations.

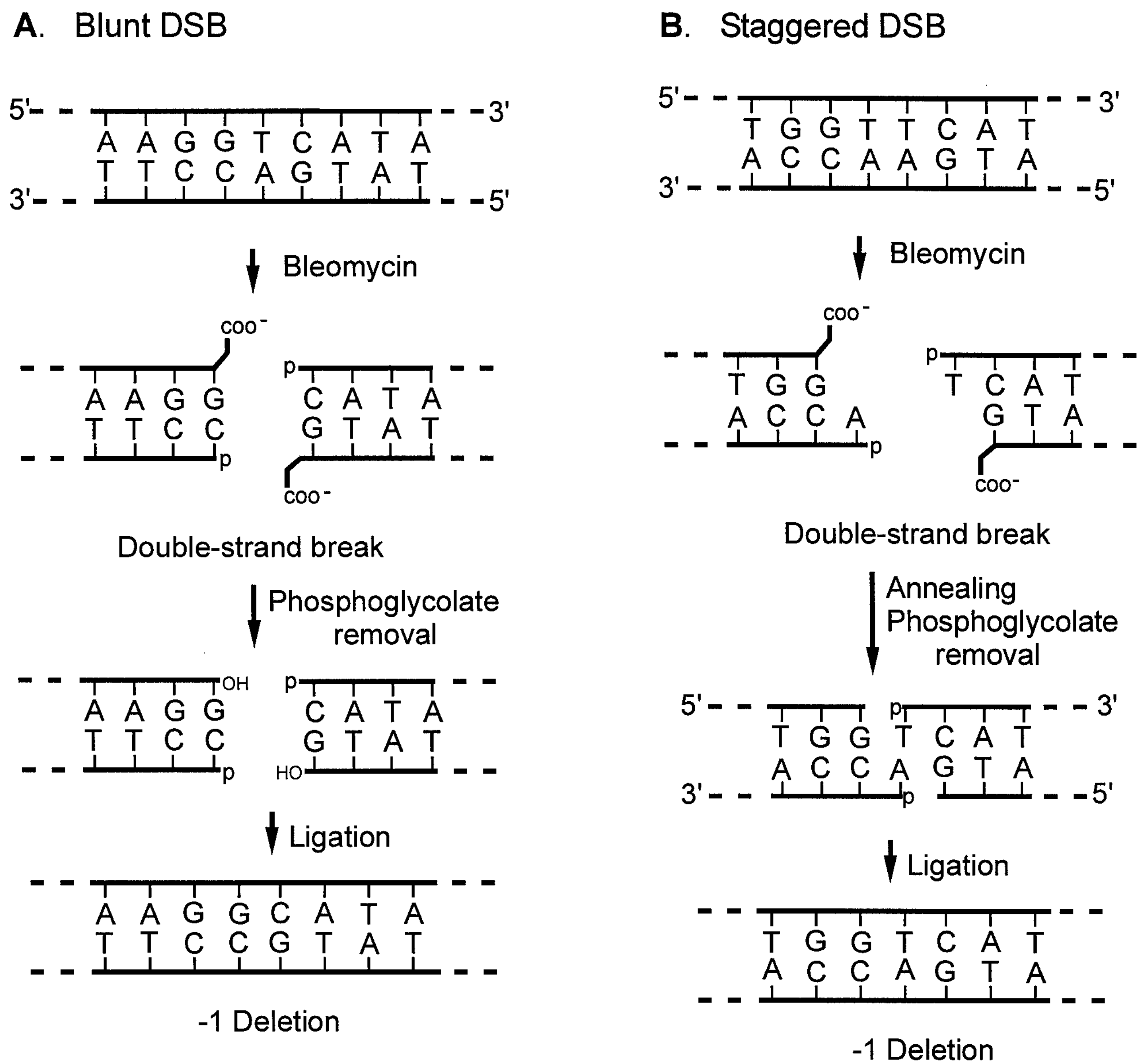


Figure 15. Proposed mechanism for formation of -1 deletions at sites of bleomycin-induced DSBs. In each case, bleomycin-induced cleavage results in destruction of one nucleotide in each strand, leaving a phosphoglycolate sugar fragment at each 3' terminus. At a blunt-ended break (**A**), phosphoglycolate removal followed by blunt-end ligation will result in a -1 deletion, regardless of sequence. At a staggered break (**B**), if the 5' overhangs are complementary, annealing can occur, and phosphoglycolate removal followed by cohseive-end ligation will again give a -1 deletion. However, in the more usual case that the overhangs are noncomplementary, they will be filled in to give a blunt end (not shown), and blunt-end ligation will then restore the original sequence.

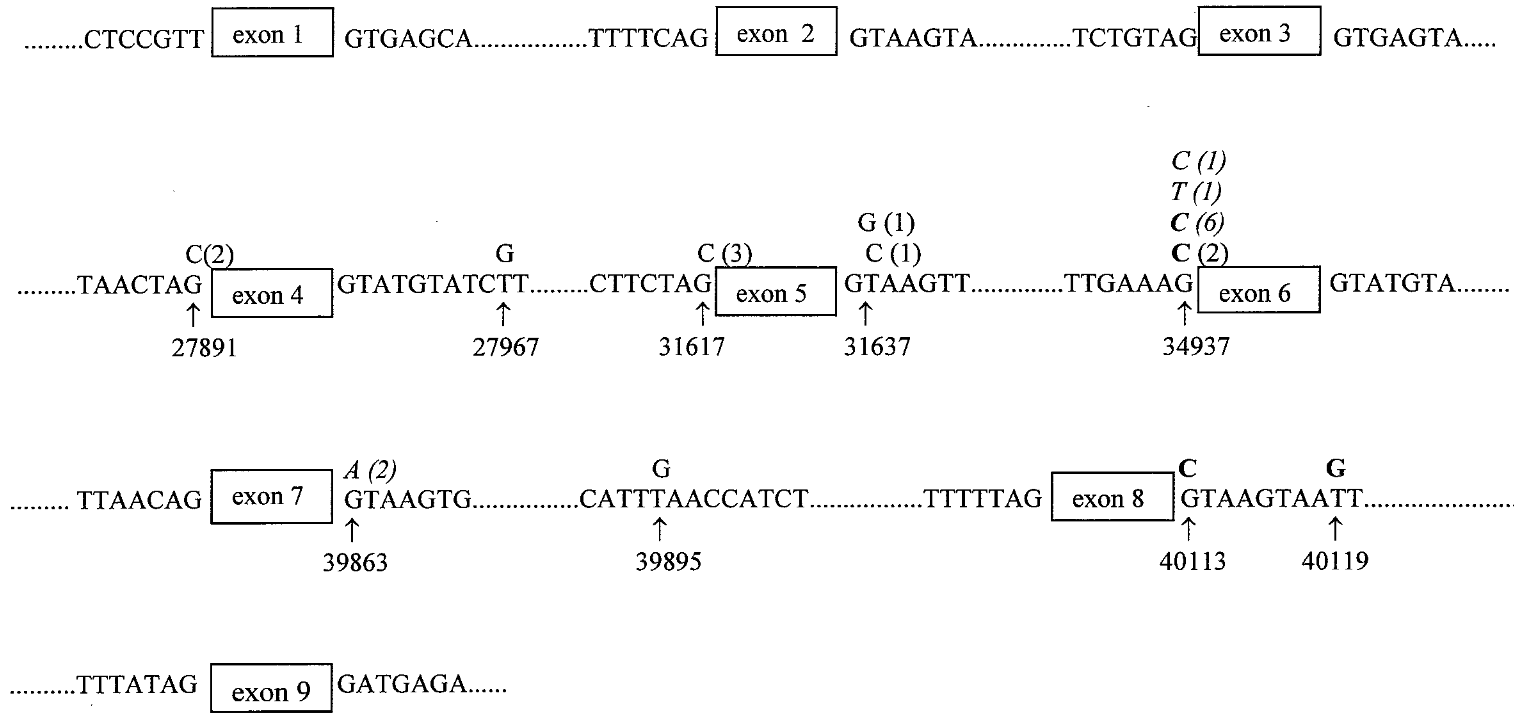


Figure 16: DNA sequence of splice junction sites for HPRT exon skipping mutations from 184B5 and 184B5 E6*tfx*C6 cell lines. The nine exons of the HPRT gene are represented by labeled boxes. The splicing junction sequences are shown for each exon. Base substitutions are shown above the nucleotides which they replaced. Bleomycin-induced mutations are represented by bold letters for the 184B5 cell line and bold italic letters for the 184B5 E6*tfx*C6 cell line. Spontaneous mutations were represented with regular letter for the 184B5 cell line and with regular italic letter for 184B5 E6*tfx*C6 cell line. The numbers inside the parentheses indicate the number of mutants that had the same sequence change.

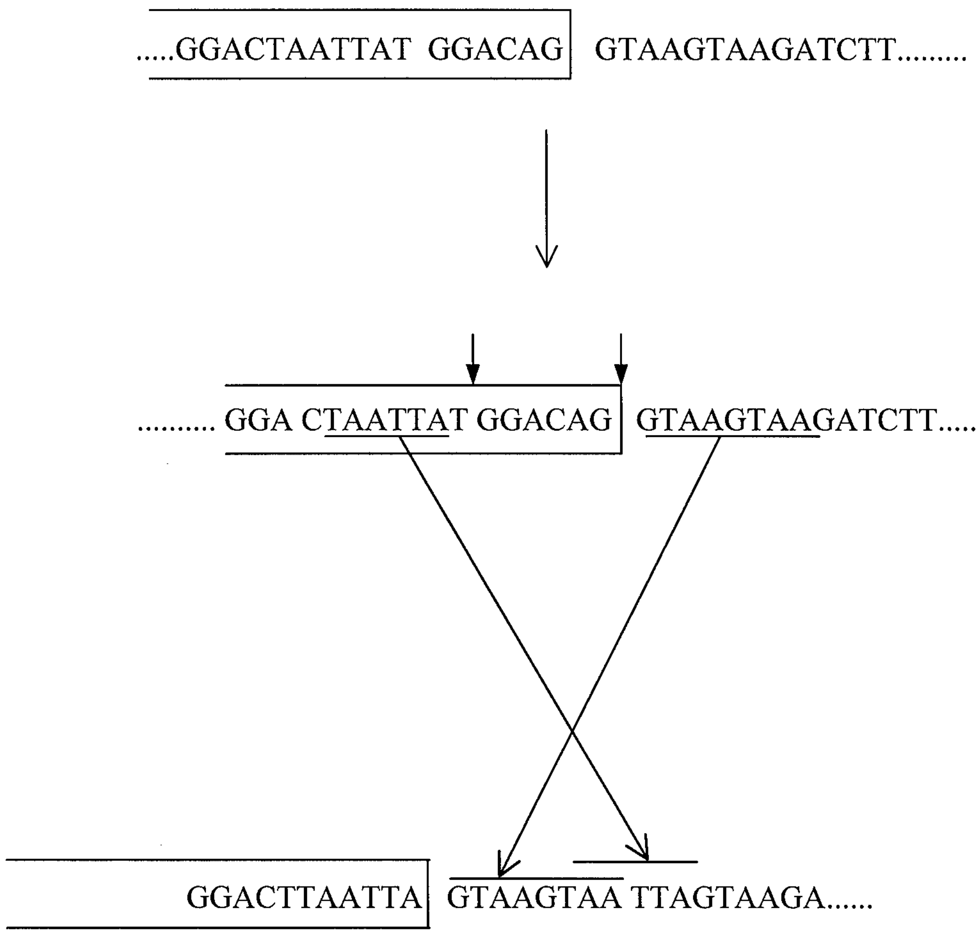


Figure 17: Small-scale rearrangement involving partial exon 2 deletion. The coding strand of HPRT cDNA is shown. The open box represents exon 2. Closed arrows indicate the apparent deletion breakpoints. Underlined nucleotides were rearranged as indicated by the open arrows. The last 6 nucleotides at 3' end of exon 2, between the two breakpoints, were lost.

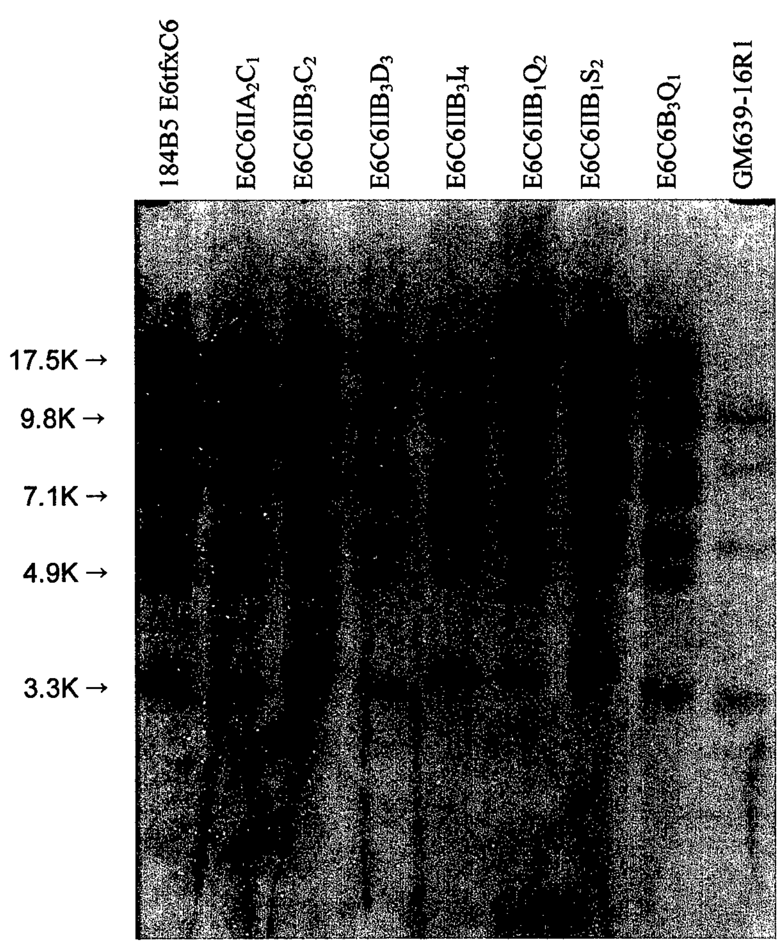
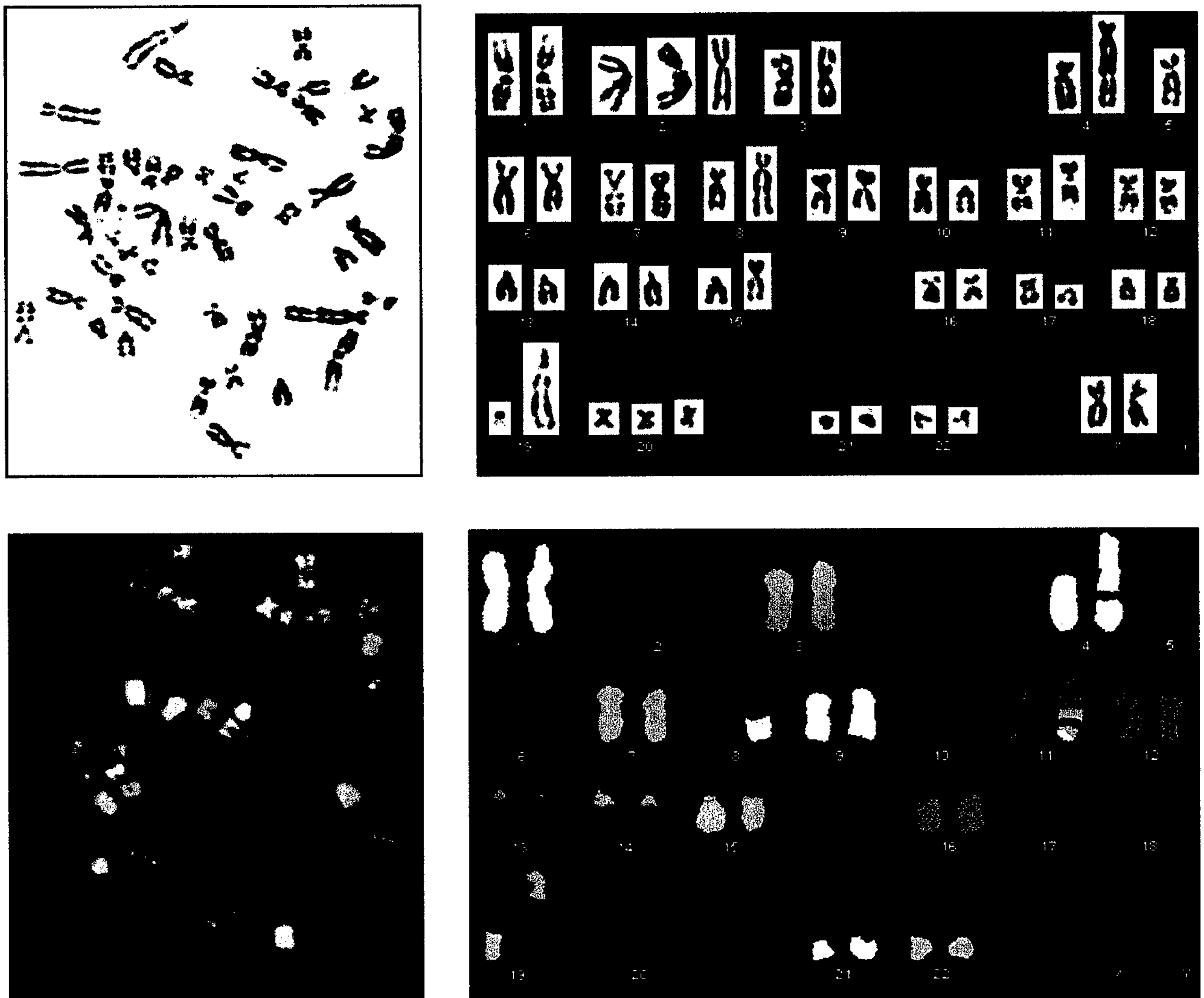


Figure 18: Southern analysis of exon-skipping mutants from 184B5 E6*tfx*C6 cell line. Restriction fragment patterns of DNA from 7 exon-skipping mutants were shown. DNA was digested with HindIII restriction enzyme. The DNA fragment pattern changes were observed in lane 2, 5 and lane 6 in comparison with the control DNA sample (lane 1). GM639-16R1 DNA was used as negative control. Molecular weight markers are in kilobases.



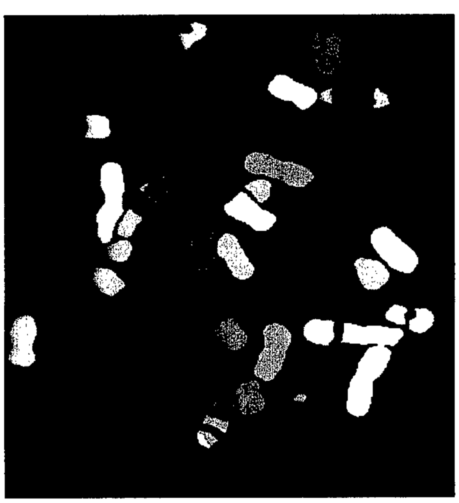
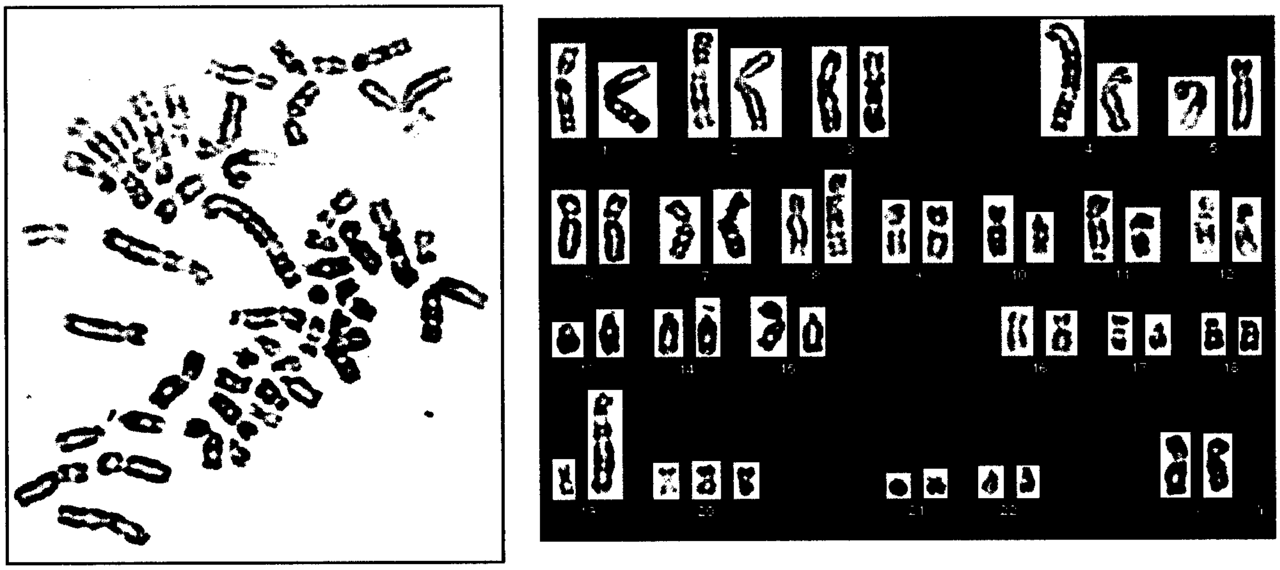
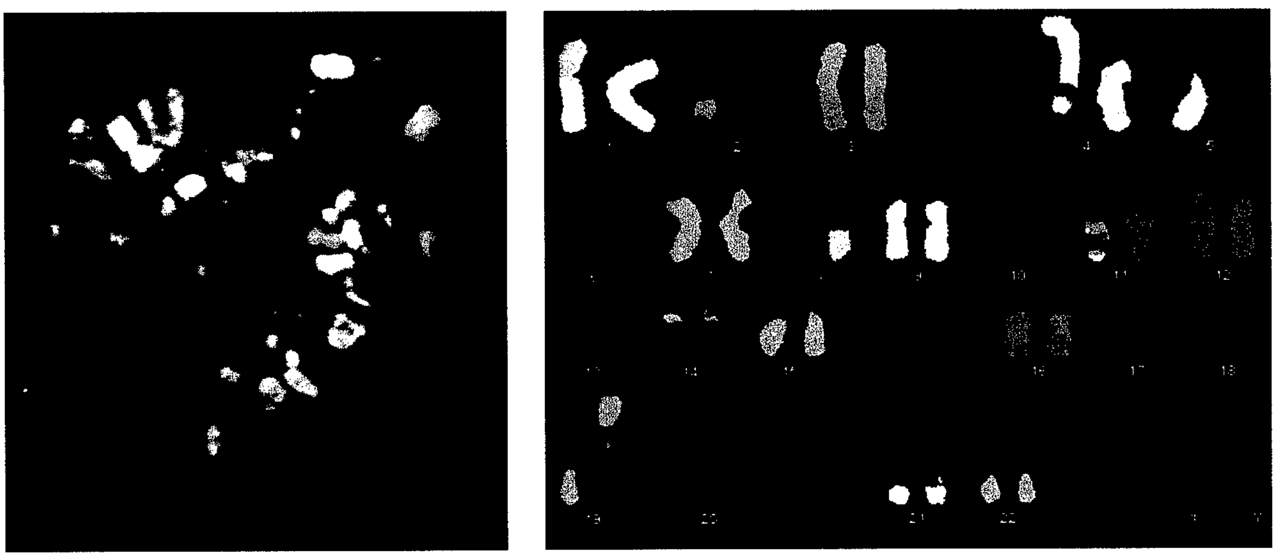


Figure 19: Spectral karyotyping of a spontaneous mutant from 184B5 cell line, A1F5. 47,XX, +der(2)t(2;5), der(4)t(1;6;4), -5, der(8)t(1;4;8), del(10), der(11)t(9;3;8;3;11), der(15)t(5;15), del(17), der(19)t(5;11;17;19), +20. The basic set of chromosome alterations carried by its parental cell line 184B5 include: +der(2)t(2;5), -5, del(10), der(15)t(5;15), del(17), der(19)t(5;11;17;19), +20.
This mutant shows specific changes: der(4)t(1;6;4), der(8)t(1;4;8), der(11)t(9;3;8;3;11).





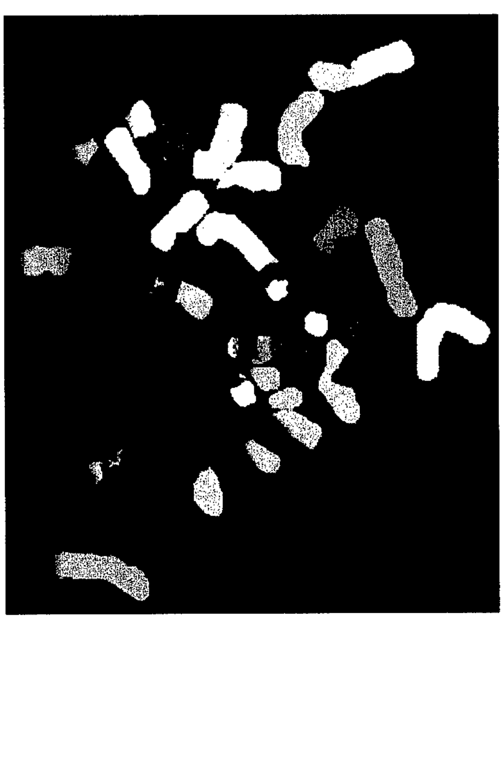


Figure 20: Spectral Karyotyping of bleomycin induced mutant from 184B5 cell line. B_21F_4. 47, XX, der(1)t(1;15), der(2)t(2;3;5), der(4)t(4;6;1;6;5), der(5)t(1;5), der(8)t(1;4;8), del(10), der(11)t(9;3;8;3;11), der(15)t(5;15), del(17), der(19) t(5;11;17;19), +20. The basic set of chromosome alterations the parental line carries include der(15)t(5;15), del(17), der(19) t(5;11;17;19), del(10), +20. The mutant also has specific chromosome alterations: der(1)t(1;15), der(2)t(2;3;5), der(4)t(4;6;1;6;5), der(8)t(1;4;8), der(11)t(9;3;8;3;11).

Table 1: Summary of cell cycle distribution after X-ray irradiation

	184B5 cell line			184B5 E6tfxC6 cell line		
	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
Normal cell cycle	56.5	20.9	22.7	42	26	32
Irradiated with 6 Gy X-ray	40.4	14.4	45	11.2	31.5	57
Cultured in medium with 50ng/ml nocodazole	13.8	17.5	68.5	0.2	0	99.8
Irradiated with X-ray and cultured in medium with 50 ng/ml nocodazole	28.8	16	55	4.7	3.9	91.4

Table 2: Average cell survival and mutation frequency.

	184B5 cell line				184B5 E6tfxC6 cell line			
Dose (μg/ml)	Log Survival (± s.e)	n	mutation frequency (± s.e)	n	Log Survival (± s.e)	n	mutation frequency (± s.e)	n
0.0	0.0 ± 0.0	22	2.1 ± 0.76	7	0.00 ± 0.0	12	2.0 ± 0.7	8
1.5	-0.4 ± 0.11	4			-0.52 ± 0.09	4		
2.5	-0.39 ± 0.05	24	9.9 ± 2.0	10	-0.45 ± 0.06	8	8.8 ± 3.0	4
5.0	-0.55 ± 0.08	24	9.6 ± 2.1	11	-0.50 ± 0.05	14	9.6 ± 2.8	8
11.0	-0.86 ± 0.16	8			-1.13 ± 0.13	4		

Table 3. Comparison of spontaneous and bleomycin-induced mutations

	184B5 cell line				184B5 E6*tfx*C6 cell line			
type of mutations	**Spontaneous mutations**	**(%)**	**Bleomycin-induced mutations**	**(%)**	**Spontaneous mutations**	**(%)**	**Bleomycin-induced mutations**	**(%)**
Base substitutions	21	63.6	26	50	16	59.3	27	47.4
-1 deletions	0	0	4	7.7	0	0	9	15.8
Small deletions	2	6.1	9	17.3	5	18.5	5	8.8
exon skipping	9	27.3	11	21.2	6	22.2	14	24.5
Rearrangements	0	0	1	1.9	0	0	0	0
No change	1	3.0	1	1.9	0	0	2	3.5
Total	33	100	52	100	27	100	57	100

Table 4: Bleomycin-induced deletions in the 184B5 and 184B5 E6*tfx*C6 cell lines.

Mutant Strain	Number of base pairs deleted	Position in HPRT cDNA	Sequence[a]
184B5			
Exon 2			
IIB_42C_1	1	41	TGATGATG a ACCAGGTT[a]
IIB_42C_2	1	53	AGGTTATG a CCTTGATT
IVB_31E_3	6	79 ~ 85	CCTAAT cattatg CTGAGGA
IVB_31B_1	6	79 ~ 85	CCTAAT cattatg CTGAGGA
Exon 3			
VB_32D_1	1	180	GGAGGCCA t CACATTGT
$IIIB_62I_1$	7	141 ~ 147	GACTGA acgtctt GCTCGAG
$IIIB_32E_1$	7	141 ~ 147	GACTGA acgtctt GCTCGAG
$IIIB_31D_1$	9	141 ~ 149	GACTGA acgtcttgc TCGAGA
VB_32I_1	7	141 ~ 147	GACTGA acgtctt GCTCGAG
Exon 6			
$IIIB_61J_2$	1	452	CTTGGTCA g GCAGTATA
Exon 9			
VB_31G_1	7	616 ~ 622	CATGTT tgtgtca TTAGTGA
184B5 E6*tfx*C6			
Exon 1			
IIB_3F_1	1	23	CCCTGGCG t CGTGATTA
IIB_3Q_2	1	23	CCCTGGCG t CGTGATTA
Exon 2			
IIB_1Q_1	1	88	ATTATGCT g AGGATTTG
IIB_3P_2	1	35	GATTAGTG a TGATGAAC
IIB_3M_3	1	35	GATTAGTG a TGATGAAC
IIB_3G_3	6	129 ~ 134	AATTAT ggacag GACTGAA
Exon 6			
IIB_3N_1	1	453	TTGGTCAG g CAGTATAA
Exon 7			
IIB_3A_2	1	523	TTGGATAT a AGCCAGAC
IIB_3C_1	1	502	TGAAAAGG a CCCCACGA
IIB_1I_1	2	526 ~ 527	GATATAAG cc AGACTTTG
IIB_3B_2	2	526 ~ 527	GATATAAG cc AGACTTTG
IIB_3I_2	2	526 ~ 527	GATATAAG cc AGACTTTG

a Sequence shown are coding strand. Lowercase letters indicate the nucleotide(s) that have been deleted.

Table 5. Targeting of -1 deletions to potential bleomycin cleavage sites

	Target sites[a]	Nontarget sites	Significance
Available sites[b]	146	511	
-1 deletions, 184B5 cells	3	1	$p < 0.03$
-1 deletions, 184B5-E6*tfx*C6 cells	7	4	$p < 0.005$
-1 deletions, Both cell lines	10	5	$p < 0.0005$

a. Potential sites of bleomycin-induced cleavage in *HPRT* coding sequences. Includes all G-C and G-T sites, plus sites of repeated nucleotides in sequences of the form GCCC... or GTTT..., since a -1 deletion at such a site cannot be unambiguously assigned to a single nucleotide position.

b. All nucleotide positions in the *HPRT* coding sequence.

Table 6: Consistent cytogenetic findings.

Cell Lines:	Parental Lines		Bleomycin-induced *HPRT* Mutants of 184B5						Spontaneous Mutant
Consistent Alteration	**184B5 (46)**	**184B5 E6*tfx*C6 (46)**	**184B5 $B_2 2F_1$ (46)**	**184B5 B_2E_1 (46)**	**184B5 $B_4 2B_1$ (46)**	**184B5 $B_2 2G_1$ (47)**	**184B5 $B_2 2D_1$ (46)**	**184B5 $B_2 1I_4$ (47)**	**184B5 A_1C_1 (48)**
der(2)t(2,5)(q33;q31)	+	+	+	+	+	+	+	+	+
der(4)t(1;4)(q21;q35)	+	+	+	+	+	+	+	+	+
-5	+	+	+	+	+	+	+	+/-	+/-
der(8)t(1;8)(q25;q24.3)	+	+	+	+	+	+	+	+	+
del(10)(p11.2)	+	+	+	+	+	+	+	+	+
ins(11;8)(q23;?)	+	+	+	+	+	+	+	+	+
der(15)t(5;15)(p12;p11.2)	+	+	+	+	+	+	+	+	+
del(17)(p11.2)	+	+	+	+	+	+	+	+	+
*der(19)t(5;11;17;19) (q13;?;q21q25;p13.3)	+	+	+	+	+	+	+	+	+
+20	+	+	+	+	+	+	+	+	+
?del(1)(q42)	-	-	-	-	+	-	-	-	+/-

Table 7: Incidental cytogenetic findings.

Type of Cell Line	Cell line	Cytogenetic findings
Parental Line	184B5 (46)	[+mar(3)]
Parental Line	184B5 E6tfxC6 (46)	der(7) t(5;7)(p13;q11.2), der(15) t(7;15)(q11.2;p11.2) [may also have 5 from der(15) t(5;15)]
Bleomycin-induced Mutant	184B5 B_42B_1 (46)	? 6's, add(9)(p2?4)
Bleomycin-induced Mutant	184B5 B_22G_1 (47)	[+mar], (similar to i(10))
Bleomycin-induced Mutant	184B5 B_22D_1 (46)	del(1)(p11), [+2[2]], [+mar[2]], [+mar[1]]
Bleomycin-induced Mutant	184B5 B_21I_4 (47)	[+/-?;(5)(q10)], [+mar]
Spontaneous Mutant	184B5 A_1C_1 (48)	+i(10)(q10), [+mar[1]], [+der(8)[1]]

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MS #1

Influence of ionizing radiation on proliferation, c-*myc* expression and the induction of apoptotic cell death in two breast tumour cell lines differing in p53 status

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Abstract.

Purpose: To determine the capacity of ionizing radiation to inhibit proliferation, to suppress c-*myc* expression and to induce apoptotic cell death in the p53 wild-type MCF-7 cell line and the p53 mutated MDA-MB231 cell line.

Materials and methods: Growth inhibition and cell killing were determined by cell number and trypan blue exclusion. Apoptosis was assessed through cell morphology and fluorescent end-labelling. c-*myc* expression was monitored by Northern blotting.

Results: Inhibition of cell proliferation by ionizing radiation was similar in both cell lines. MDA-MB231 cells accumulated in G_2 while MCF-7 cells accumulated in both the G_1 and G_2 phases of the cell cycle after irradiation. There was no evidence of apoptosis in either cell line. In MCF-7 cells, growth inhibition correlated closely with an early dose-dependent suppression of c-*myc* expression; in MDA-MB231 cells, there was no correspondence between growth inhibition and a transient, dose-*independent* reduction in c-*myc* message.

Conclusions: These findings suggest that in the absence of classical apoptotic cell death, radiosensitivity is not predictably related to the p53 status of the cell. While both p53 and c-*myc* may be linked to the DNA damage response pathway, neither p53 nor c-*myc* are essential for growth arrest in response to ionizing radiation.

1. Introduction

The p53 tumour suppressor gene is one of the primary cellular factors which determines the nature of growth arrest and/or cell death in response to ionizing radiation (Kastan *et al.* 1991, Kuerbitz *et al.* 1992, Tishler *et al.* 1993, Zhan *et al.* 1993). An increase in the level of the p53 protein in irradiated cells and the consequent up-regulation of the cyclin dependent kinase inhibitory protein, $p21^{waf1/cip1}$ appear to be critical components of G_1 arrest (Kastan *et al.* 1991, Zhan *et al.* 1993, El Deiry *et al.* 1994, Bae *et al.* 1995, Gudas *et al.* 1995). Functional p53 is also frequently required for the induction of programmed or apoptotic cell death (Yonish-Rouach *et al.* 1991, Ramqvist *et al.* 1993), although there is also unequivocal evidence for p53 independent apoptosis (Jarvis *et al.* 1994, Bracey *et al.* 1995). The dual roles of p53 in G_1 arrest and apoptosis suggest that the p53 status of the cell could be a fundamental component of radiosensitivity, particularly in cells which undergo radiation-induced apoptosis (Lowe *et al.* 1993a, 1994).

Studies in this laboratory have demonstrated that MCF-7 breast tumour cells fail to undergo apoptotic cell death in response to the topoisomerase II inhibitors, doxorubicin (Fornari *et al.* 1994, 1996) and teniposide (unpublished data). Studies by other investigators support the concept that MCF-7 cells are relatively refractory to DNA-damage induced apoptosis (Oberhammer *et al.* 1993, Zhan *et al.* 1994). Consequently, while mutations in p53 have been reported in breast cancer (Elledge and Alfred 1994), the role of p53 in determining the nature of the cellular response to ionizing radiation could be limited in those breast tumour cells which fail to undergo apoptosis in response to DNA damage.

In addition to p53, the oncogene, c-*myc*, may also play a central role in the proliferative activity of breast tumour cells (Watson *et al.* 1991, Shiu *et al.* 1993). c-*myc* is reported to be deregulated or overexpressed in many clinical breast tumour samples (Escot *et al.* 1986, Kreipe *et al.* 1993) while amplification of c-*myc* is associated with early relapse and poor response in breast tumour cells (Matiani-Constantini *et al.* 1988). The c-*myc* gene could influence the response of breast tumour cells to ionizing radiation through its role in the transition between the cell proliferation and senescence (Seth *et al.* 1993, Karn *et al.* 1989, Shichiri *et al.* 1993). The c-*myc* protein and one of its downstream targets, ornithine decarboxylase (Bello-Fernandez *et al.* 1993)—in conjunction with p53- have also been implicated in the induction of apoptosis (Evan *et al.* 1992, Henneking and Eick, 1994, Packham and Cleveland 1995). Finally, there

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is evidence that c-*myc* expression can be regulated by p53 at the level of its promoter (Moberg *et al.* 1992, Levy *et al.* 1993).

Previous studies from this laboratory in the p53 wild-type MCF-7 human breast tumour cell line have demonstrated the suppression of c-*myc* expression by various topoisomerase II inhibitors (Gewirtz *et al.* 1993, Bunch *et al.* 1994, Fornari *et al.* 1996), agents which produce transient strand breaks in DNA (Osheroff 1989, Chen and Liu 1994). In these studies, the early, concentration-dependent effects on c-*myc* expression were predictive of growth inhibition (measured 72 h after drug exposure). One focus of the present studies was to determine whether ionizing radiation, which also induces DNA damage, could suppress c-*myc* expression in MCF-7 breast tumour cells. Studies using the p53 mutated MDA-MB231 cells were designed to determine if ionizing radiation could suppress c-*myc* expression in a breast tumour cell in the absence of functional p53. We were further interested in determining whether ionizing radiation, like doxorubicin and teniposide (Fornari *et al.* 1994, 1996 and unpublished data), would fail to induce apoptotic cell death in MCF-7 cells as well as in the MBA-MB231 breast tumour cell line. Finally, we compared the antiproliferative effects of ionizing radiation in MCF-7 cells with those in MDA-MB231 cells in order to determine how a mutation in p53, which abrogates G_1 arrest, might influence the capacity of ionizing radiation to interfere with breast tumour cell growth.

2. Materials and methods

2.1. *Probes and constructs*

The c-*myc* probe, an EcoR1/Clal fragment of PMC41 3RC containing the third exon of the human c-*myc* gene (Dalla-Favera *et al.* 1982), was generously provided by Dr Eric Westin of the Medical College of Virginia. The GAPDH probe, a 780bp PstI/Xba I cDNA fragment from a pBR322 vector, was obtained from American Type Culture Collection (Rockville, MD, USA).

2.2. *Cell lines*

The MCF-7 breast tumour cell line was kindly provided by the laboratory of Dr Kenneth Cowan at the National Cancer Institute (Bethesda, MD, USA). The MDA-MB231 cell line was obtained through ATCC. Cells were maintained in Dulbecco's minimal essential media (Hazelton Research Products, Denver, PA, USA) supplemented with 5% foetal calf serum (Life Technologies, Grand Island, NY, USA), 5% defined bovine serum (Hyclone Laboratories, Logan, UT, USA) glutamine (29·2 mg/100 ml), amphotericin B (5 μg/ml) (Sigma Chemical Co.), and penicillin/streptomycin (0·5 ml/100 ml) (Whittaker Bioproducts, Walkersville, MD, USA).

2.3. *Determination of cell number and the antiproliferative activity of ionizing radiation*

Viable cell number was determined based on exclusion of trypan blue dye at intervals of 24, 48 and 72 h after irradiation. In order to distinguish between growth arrest and cell killing, cell numbers were determined at 24-h intervals after irradiation and compared with the number of control cells at the initiation of the study. For assessment of antiproliferative activity, cell numbers were compared in control cells and irradiated cells after 72 h.

2.4. *Clonogenic analysis*

MCF-7 cells in 25-cm^2 T flasks were irradiated, washed once in sterile media, and released from the flasks by incubation with trypsin (0·05 mg/ml)/EDTA (0·02 mg/ml) for 5 min at 37°C. After collection, cells were plated in triplicate at 10^3 cells/ml for each condition, and incubated at 37°C in 5% CO_2 for 10–14 days. The cells were 'fixed' with 100% methanol, plates were air-dried for 1–2 days and stained with 0·1% crystal violet. Colonies (a group of aggregated cells numbering >50) were counted; values are presented as a fraction of the growth of untreated colonies.

2.5. *Cell cycle analysis*

At appropriate intervals after irradiation, DNA content per cell was determined by cytofluorimetry using a Beckton-Dickson FACScan Model FC.

2.6. *Light microscopic analysis of cell morphology*

MCF-7 or MDA-MB231 breast tumour cells in 75 cm^2 T flasks (Costar) were irradiated and incubated for an additional 72 h. At appropriate intervals the medium was aspirated, the cells washed with ice-cold phosphate buffered saline (pH 7·4) and released from flasks by incubation in trypsin (0·05 mg/ml)/EDTA (0·02 mg/ml) for 5 min at 37°C. The cells were collected in ice-cold phosphate-buffered saline (pH 7·4) and centrifuged at 4°C. After resuspension, the cells were deposited on cytocentrifuge slides and stained with a 20% Wright-Giemsa stain (Fornari *et al.* 1996).

2.7. *Terminal end labelling (TUNEL) assay*

The method of Gavrielli *et al.* (1992) was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells.

2.8. *Gene expression by Northern blotting*

Message expression was determined in adherent cells by standard Northern blotting. RNA was isolated from cells using the RNA-STAT procedure as described by the manufacturer (Tel-Test B Inc., Friendswood, TX, USA). RNA samples (10 μg) were electrophoresed in 1% agarose—2·2M formaldehyde gels (Thomas *et al.* 1980) and transferred to nylon filters. Hybridization was performed according to the method of Maniatis *et al.* (1982).

2.9. *Error analysis*

Statistical analyses were performed using the Student's t test. $p \leqslant 0{\cdot}05$ was considered to be of statistical significance.

3. Results

3.1. *Influence of ionizing radiation on the proliferation of MCF-7 and MDA-MB231 breast tumour cells*

The influence of ionizing radiation on the proliferation of the p53 wild-type MCF-7 breast tumour cells and the p53-mutated MDA-MB231 cells was evaluated by comparing the number of control and irradiated cells 72 h after exposure to various doses of ionizing radiation. In separate studies (not shown) the growth inhibition assay was shown to correspond closely with loss of clonogenicity. As indicated in Figure 1, over the range 0·5–10 Gy, ionizing radiation produced a dose-dependent reduction in cell proliferation. While the MDA-MB231 cells appeared to be slightly more sensitive at doses of 0·5 and 1 Gy, the effects of ionizing radiation were essentially identical over the dose range between 2·5 and 10 Gy in the MCF-7 and MDA-MB231 cells.

3.2. *Influence of ionizing radiation on progression through the cell cycle*

To confirm that the mutated *p53* gene in MDA-MB231 cells prevents arrest in G_1, cell cycle distribution was assessed after exposure of MDA-MB231 cells to 6 Gy ionizing radiation, a dose which produces an approximately 80–90% reduction in cell proliferation. As shown in Figure 2, within 24 h after irradiation of MDA-MB231 cells, the G_2 fraction increased from 13·4 to 55%, consistent with arrest in G_2 M while the S phase fraction declined from 25 to 19%. There was no indication of G_1 arrest in the MDA-MB231 cells as the G_0/G_1 fraction declined from 59 to 20%. A population of sub G_0 cells (approximately 15% of the total) as well as a small fraction of polyploid cells were evident within 24 h.

The influence of irradiation on cell-cycle distribution of MCF-7 cells is also indicated in Figure 2. MCF-7 cells demonstrated evidence of arrest in both G_1 and G_2, similar to the report by Fan *et al.* (1995). At 24 h, the G_1 population increased from 59 to 79% and the G_2/M population increased from 13 to 17%, while the S phase population declined from 28 to 5%. Similar to the studies with MDA cells, there was evidence of polyploidal cells; however, there was no evidence of a sub G_0 population even at 48 and 72 h after irradiation (not shown).

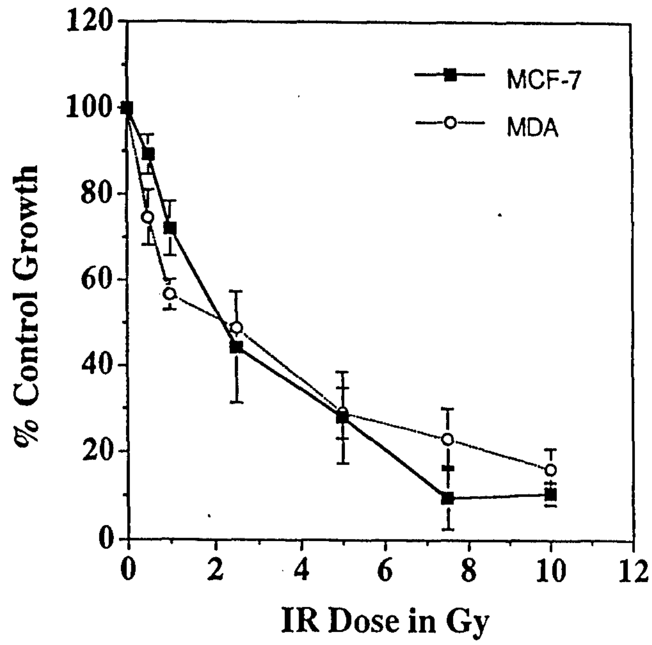


Figure 1. Influence of ionizing radiation on proliferation of MCF-7 and MDA-MB231 breast tumour cells. The two breast tumour cell lines were irradiated as described in the Materials and methods and growth inhibition was calculated based on the relative growth rates of control and irradiated cells after 24 h—where growth of control cells is taken as 100%. Values represent means ± standard errors for four replicate experiments (MCF-7) and three replicate experiments (MDA-MB231).

3.3. *Discrimination between the effects of ionizing radiation on cell growth and cell death*

Ionizing radiation has been shown to induce apoptotic cell death in a number of different experimental tumour cell lines, usually by 24 h, but always within 48 h (Warters *et al.* 1992, Lowe *et al.* 1993, Stephens

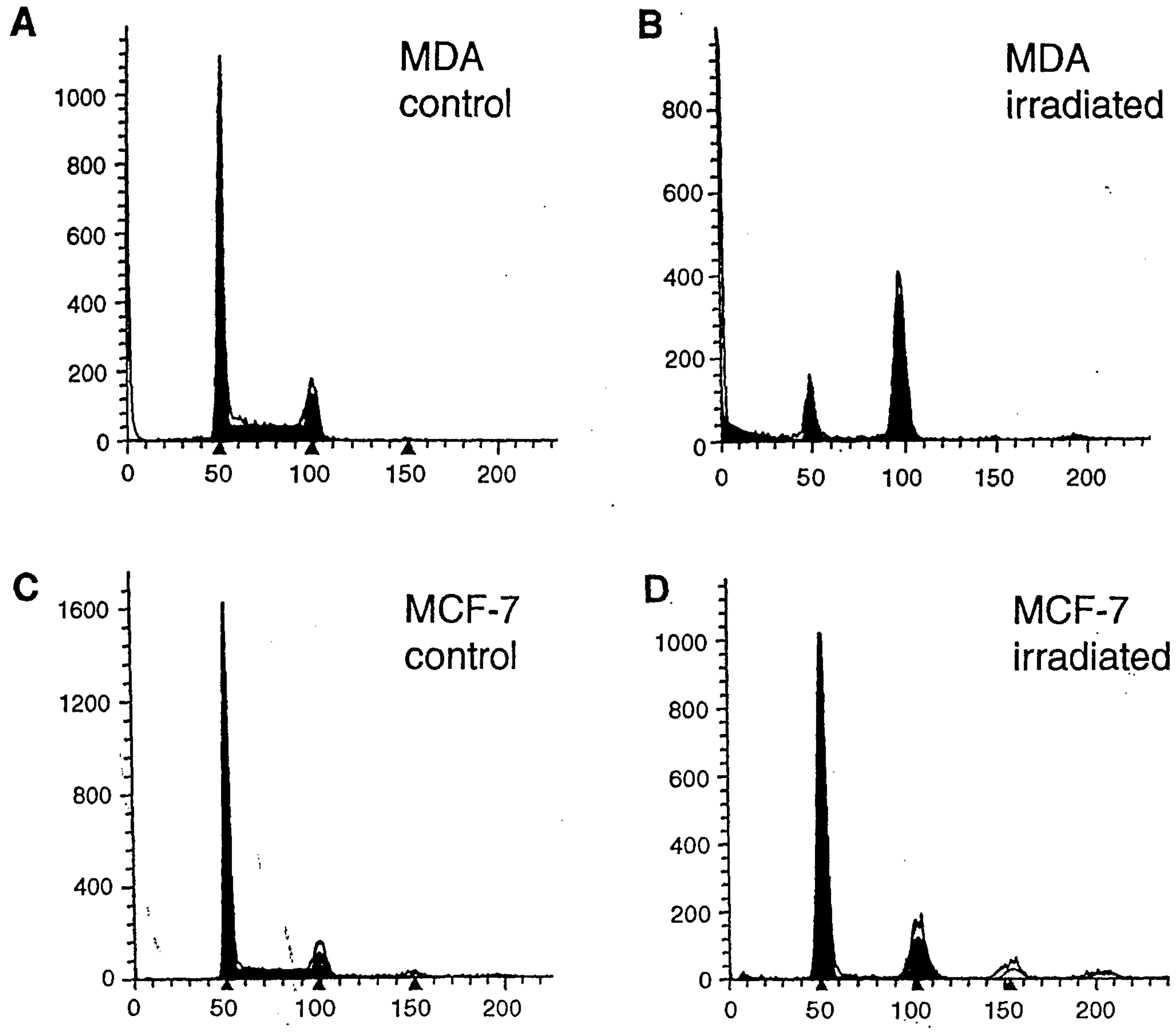


Figure 2. Influence of ionizing radiation on cell cycle traverse. Cells were isolated for determination of cell cycle distribution at 24 h after exposure to 6 Gy ionizing radiation.

et al. 1993, Langley *et al.* 1994, Radford *et al.* 1994, Seki *et al.* 1994, Zhan *et al.* 1994, Alridge *et al.* 1995, Ling *et al.* 1995, Palayoor *et al.* 1995, Zhen *et al.* 1995). Consequently, studies were performed to determine whether ionizing radiation produced cell death (either by apoptosis or necrosis) in breast tumour cells. Figure 3 presents an analysis of cell number at 24 h intervals over 96 h after irradiation and demonstrates growth arrest in both the MCF-7 and the MDA-MB231 cells. While there is some variability in the data, there was no clear evidence of a reduction in absolute cell number (when compared with the cell number at the initiation of the experiment) over the time frame of these studies. Although it has been established that irradiated cells may traverse (at least) one additional cell cycle prior to growth arrest (Chang and Little 1991), both the MCF-7 cells and the MDA-MB231 cells demonstrated a limited capacity for replication even during the first 24-h post-irradiation interval. Consequently, at least over the time frame of 96 h, the predominant effect of ionizing radiation appears to be growth arrest.

3.4. *Absence of apoptotic cell death in irradiated breast tumour cells*

Our laboratory has previously reported on the relative refractoriness of MCF-7 breast tumour cells to apoptotic cell death in response to the DNA

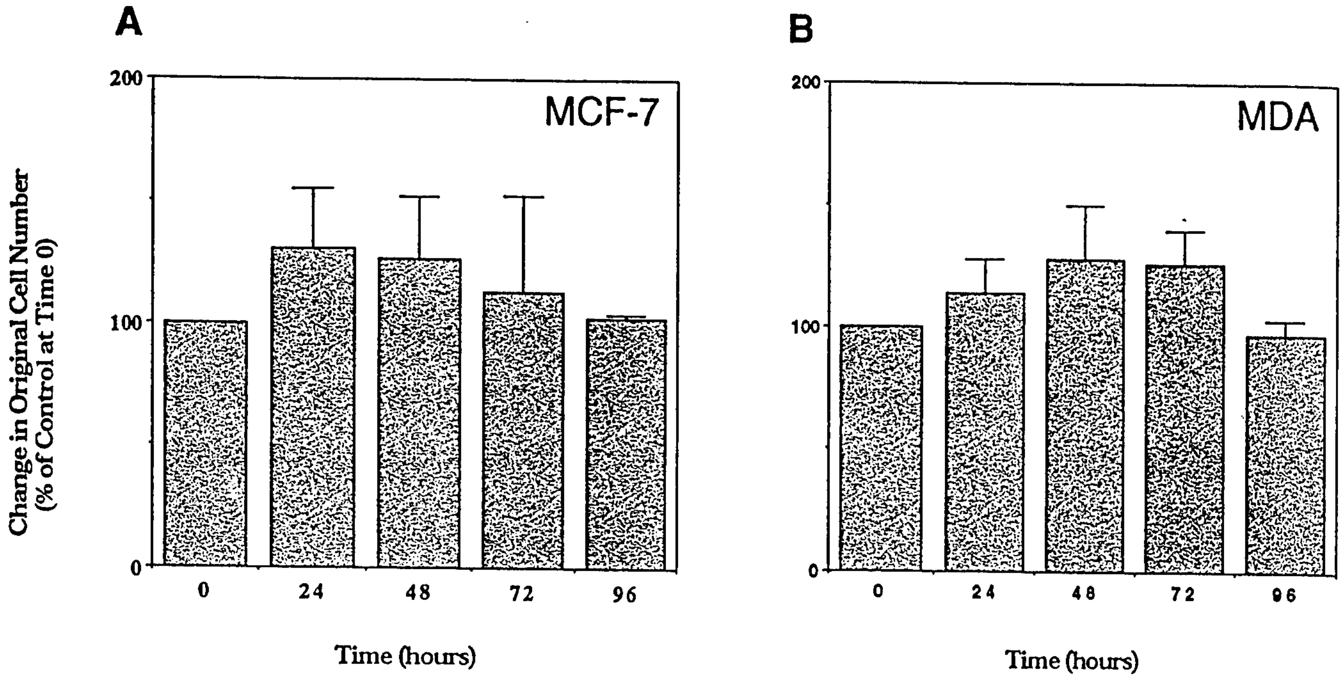


Figure 3. Determination of cell number after irradiation. MCF-7 cells or MDA-MB231 cells were exposed to 10 Gy and the number of adherent cells was determined in irradiated cells at 24-h intervals. Data represent means ± standard errors for four replicate experiments for each cell line. Controls in this series of experiments represents cell number at the *initiation* of the experiment. These studies should be distinguished from those presented in Figure 1 where controls represent cell number in unirradiated flasks after 72 h of growth.

damaging agent, doxorubicin (Fornari *et al.* 1994 and unpublished results). Figure 4 presents light micrographs of MDA-MB 231 (A) and MCF-7 cells (B) at 24, 48 and 72 h after exposure to 10 Gy irradiation. Both cell lines demonstrate morphology which is clearly distorted, with the formation of multinucleate cells which are apparently the result of a failure of the cells to undergo division. However there was little evidence for apoptotic bodies or cell shrinkage which would reflect an apoptotic mode of cell death (Gerschenson and Rolello 1992). These morphological studies were supported the by fluorescent end-labelling analyses presented in Figure 5, which indicate that DNA fragmentation was barely detectable in MCF-7 and MDA-MB231 cells at 48 h after exposure to radiation doses as high as 20 Gy. Consequently, the absence of apoptosis is consistent with the induction of growth arrest rather than cell death by ionizing radiation in both MCF-7 and MDA-MB231 cells.

3.5. *Influence of ionizing radiation on c-*myc *expression in MCF-7 cells*

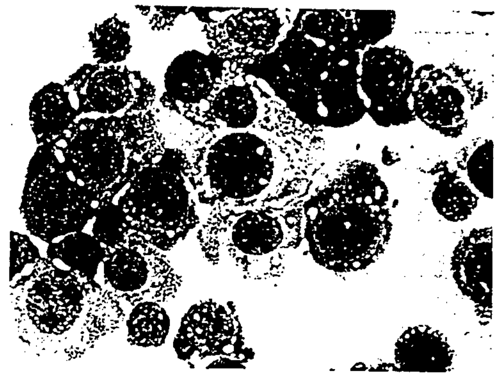
Previous work from this laboratory has demonstrated that drugs which induce strand breaks in DNA via inhibition of the religation activity of topoisomerase (Osheroff *et al.* 1989, Chen and Liu 1994), produce a time- and concentration-dependent reduction in expression of the oncogene, c-*myc*, in MCF-7 breast tumour cells (Gewirtz *et al.* 1993, Bunch *et al.* 1994, Fornari *et al.* 1996). Consequently, we were interested in determining the capacity of ionizing radiation to modulate c-*myc* expression in MCF-7 and MDA-MB231 cells.

Figure 6 presents a representative Northern blot indicating that 10 Gy of ionizing radiation produced a time-dependent reduction in c-*myc* expression in MCF-7 breast tumour cells. The lanes show the levels of c-*myc* expression at 0, 0·5 1, 2, 3, 4 and 5 h after exposure to 10 Gy ionizing radiation. The absence of radiation effects on expression of the constitutively expressed GAPDH gene is presented as a loading control. Figure 6 also presents a quantitative assessment of pooled data from four independent experiments, which indicates that a maximum decline in c-*myc* expression was observed between 3 and 4 h after irradiation. All data for c-*myc* expression was normalized to GAPDH expression. In contrast to the effects of radiation on c-*myc* expression, expression of the early response gene, c-*fos* was essentially unaltered (not shown).

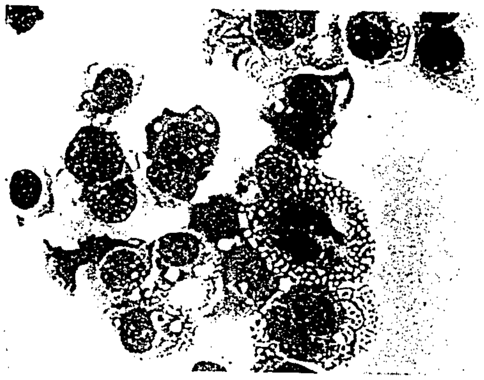
The dose-related effects of radiation on expression of c-*myc* in MCF-7 cells were analysed at 3 h, as shown in the representative Northern blot presented in Figure 7. Lane 1 presents control levels of c-*myc*

CONTROL MCF-7 CELLS

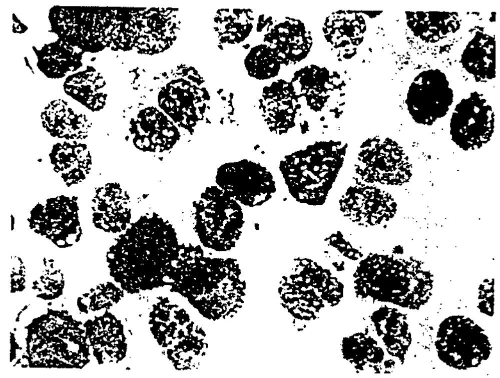
IRRADIATED 24 HOURS



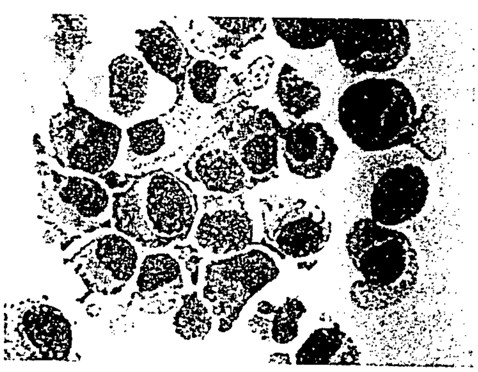
IRRADIATED 48 HOURS



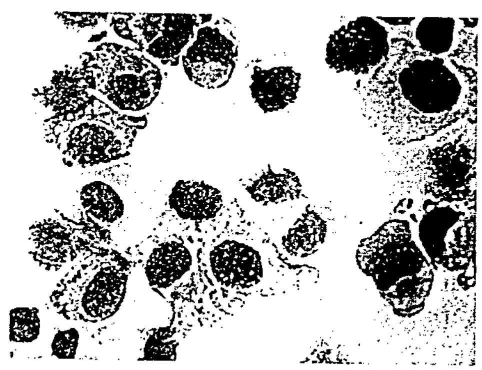
IRRADIATED 72 HOURS



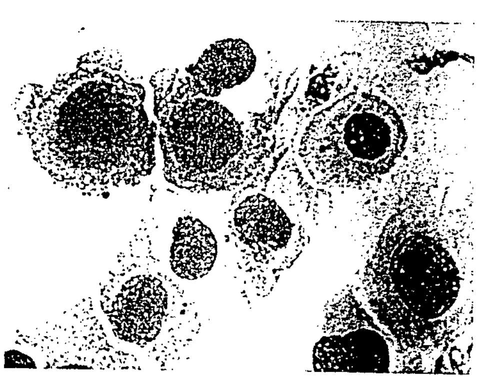
CONTROL MDA CELLS



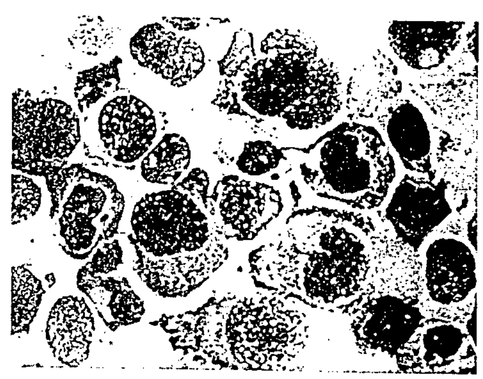
IRRADIATED 24 HOURS



IRRADIATED 48 HOURS



IRRADIATED 72 HOURS



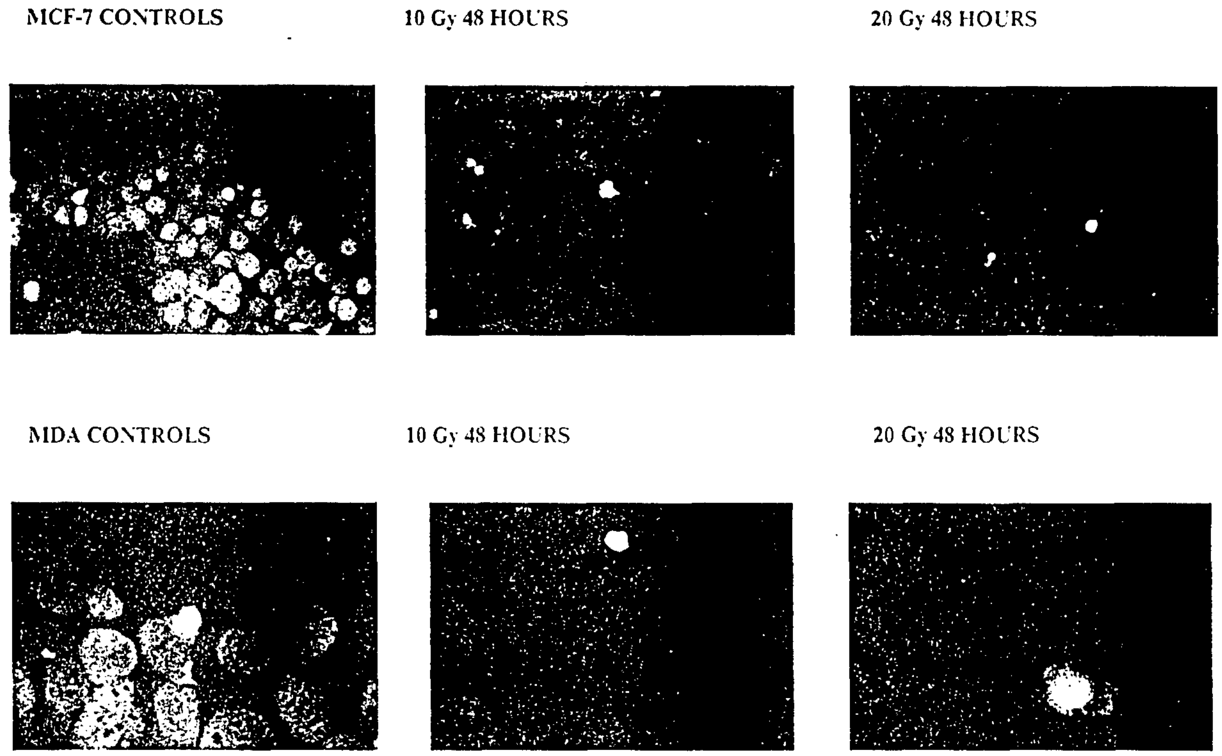


Figure 5. TUNEL assay for the induction of DNA damage by ionizing radiation in MCF-7 and MDA-MB231 breast tumour cells. MDA-MB231 and MCF-7 cells (both adherent and non-adherent) were isolated on microscope slides at the indicated times after irradiation, and DNA fragmentation was assessed by fluorescent end-labelling. (upper) Control MCF-7 and MCF-7 cells at 48 h after irradiation with doses of 10 and 20 Gy. (lower) Control MDA-MB231 and MDA-MB231 cells at 48 h after irradiation with doses of 10 and 20 Gy.

expression; lanes 2–7 demonstrate the effects of 0·5, 1, 2·5, 5, 7·5 and 10 Gy respectively on c-*myc* expression after 3 h. Expression of GAPDH, utilized as a control for loading of the gels, was essentially unchanged. Figure 7 also presents pooled data from four experiments which indicate a quantitative dose-dependent reduction of c-*myc* expression by ionizing radiation in MCF-7 breast tumour cells. (Data from the representative autorad were not included with the pooled data for dose-dependent effects of radiation on c-*myc* expression—as the pooled data were generated as the same time as the radiosensitivity data presented in Figure 1.) The more pronounced reduction in c-*myc* expression at 10 Gy in the autorad may reflect an alteration in cell radiosensitivity with continued passage of cells in culture.

3.6. *Influence of ionizing radiation on c-*myc *expression in MDA-MB231 cells*

The influence of ionizing radiation on c-*myc* expression was further evaluated in the p53 mutated MDA-MB231 cells. Figure 8 presents a representative Northern blot for c-*myc* and GAPDH expression in MDA-MB231 cells after irradiation. Ionizing radiation produced a small transient reduction in c-*myc* expression in MDA-MB231 cells which reverted to baseline levels within 3 h. This transient nature of the reduction in c-*myc* expression by ionizing radiation in MDA-MB231 cells is shown more clearly by the pooled data presented in Figure 8.

Although the suppression of c-*myc* expression in the MDA-MB231 cells appeared quite transient, it was still possible that this change in c-*myc* expression might be related to the dose-dependent inhibition of breast tumour cell growth. Consequently, we assessed the influence of various doses of ionizing radiation on c-*myc* expression after 1 h, the time when maximal suppression was observed. The representative Northern blot presented in Figure 9 indicates that, in contrast to the MCF-7 cells, ionizing radiation failed to demonstrate a dose-dependent suppression of c-*myc* expression in MDA-MB231 cells. The pooled

Figure 4. Assessment of cell morphology in irradiated MCF-7 and MDA-MB231 cells. (upper) Light microscopic analysis of MCF-7 breast tumour cells after exposure to 10 Gy ionizing radiation. Shown are control cells and cells at 24, 48 and 72 h after irradiation. (lower) Light microscopic analysis of MDA-MB231 breast tumour cells after exposure to 10 Gy ionizing radiation. Shown are control cells and cells at 24, 48 and 72 h after irradiation.

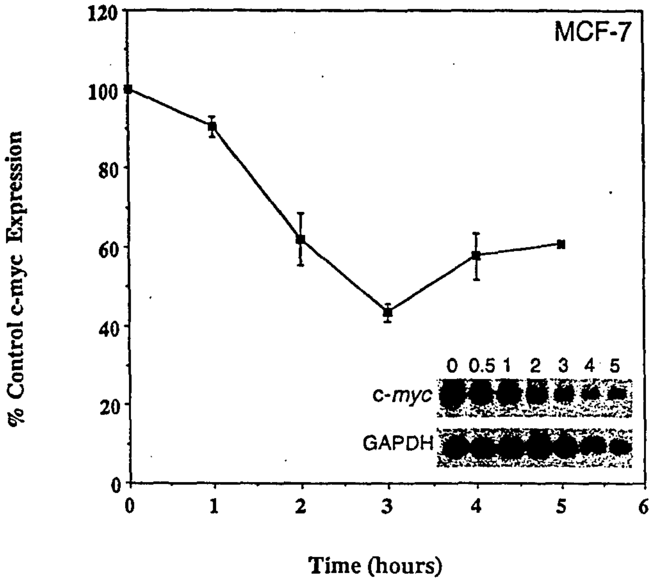


Figure 6. Analysis of c-*myc* expression at intervals after exposure of MCF-7 cells to 10 Gy ionizing radiation. Pooled data indicating the time-dependent suppression of c-*myc* expression by ionizing radiation. Values represent means ± standard errors for four replicate experiments. (inset) Representative Northern analysis indicating the time-dependent reduction in c-*myc* expression and the relatively stable expression of GAPDH after a dose of 10 Gy. Time after exposure is indicated above each lane in the autoradiograph.

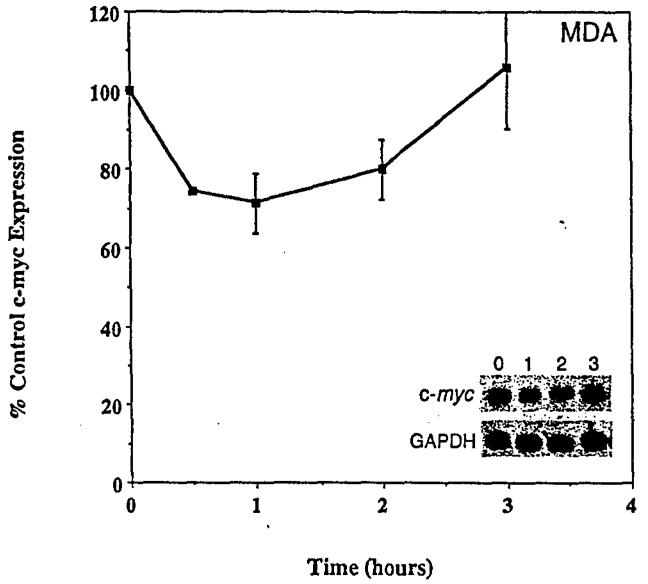


Figure 8. Analysis of c-*myc* expression at intervals after exposure of MDA-MB231 cells to 10 Gy ionizing radiation. Pooled data indicating the time-dependent suppression of c-*myc* expression by ionizing radiation. Values represent means ± standard errors for two replicate experiments. (inset) Representative Northern analysis indicating the time-dependent alterations in c-*myc* expression and the corresponding expression of GAPDH after a dose of 10 Gy. Time after exposure is indicated above each lane in the autoradiograph.

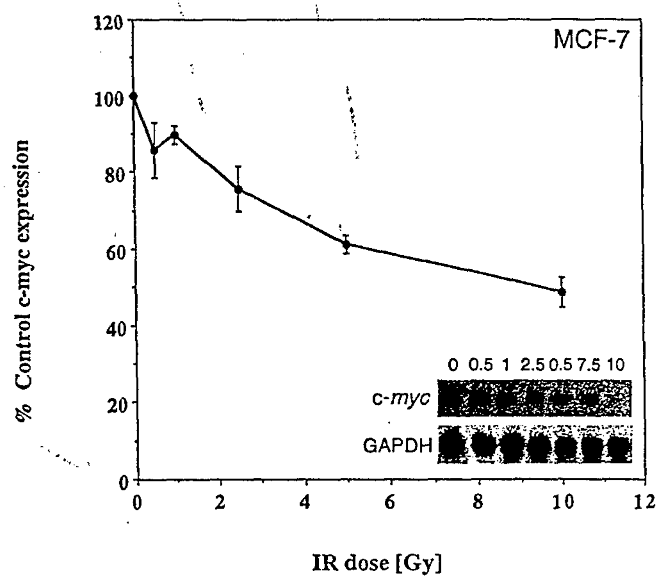


Figure 7. Analysis of dose-dependent effects of ionizing radiation on c-*myc* expression in MCF-7 cells. Quantitative representation of pooled data (mean ± standard error) from four replicate experiments. (inset) Representative Northern analysis indicating the dose-dependent reduction in c-*myc* expression and the relatively stable expression of GAPDH at 3 h. The radiation dose is indicated above each lane in the autoradiograph.

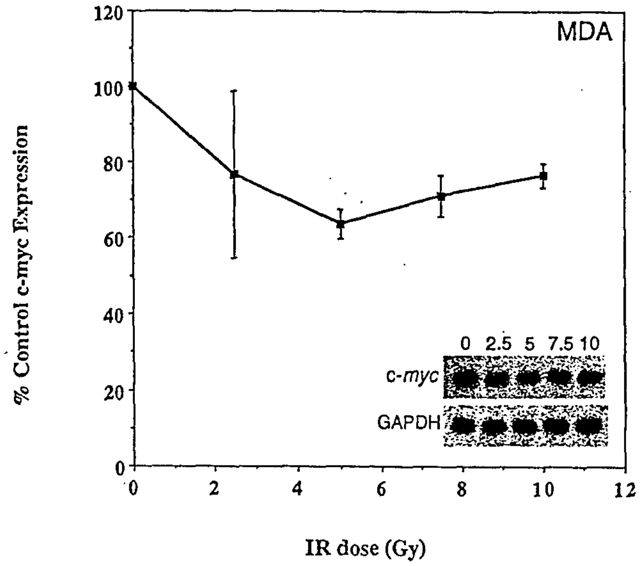


Figure 9. Influence of ionizing radiation on c-*myc* expression in MDA-MB231 cells at 1 h. Quantitative representation of pooled data (mean ± standard error) from three replicate experiments. (inset) Representative Northern analysis indicating the *absence* of a dose-dependent reduction in c-*myc* expression and the corresponding expression of GAPDH. The radiation dose is indicated above each lane in the autoradiograph.

data presented in Figure 9 indicates that the reduction in c-*myc* expression was essentially identical at all doses examined.

3.7. *Relationship between suppression of c-*myc *expression and growth inhibition by ionizing radiation in MCF-7 breast tumour cells*

We have previously reported that the suppression of c-*myc* expression by the topoisomerase II inhibitors, doxorubicin, VM-26 and m-AMSA in MCF-7 breast tumour cells corresponded closely with and was predictive of growth inhibition (Gewirtz *et al.* 1993, Bunch *et al.* 1994, Fornari *et al.* 1996). A similar relationship between growth inhibition and suppression of c-*myc* expression was evident for ionizing radiation in the MCF-7 breast tumour cell line. Figure 10 shows a strong correlation ($r^2 = 0{\cdot}93$) between the dose-dependent suppression of c-*myc* expression and of tumour cell growth (assessed 72 h after irradiation). No such relationship between radiation effects on growth and c-*myc* expression was evident for the MDA-MB231 cells (not shown).

4. Discussion

Ionizing radiation, like other modalities which induce DNA damage, has been shown to increase levels of the p53 tumour suppressor protein, an event which appears to be a prerequisite for G_1 arrest (Kastan *et al.* 1991, Kuerbitz *et al.* 1992, Tishler *et al.* 1993, Zhan *et al.* 1993, Bae *et al.* 1995, Gudas *et al.* 1995). A number of studies have suggested that the

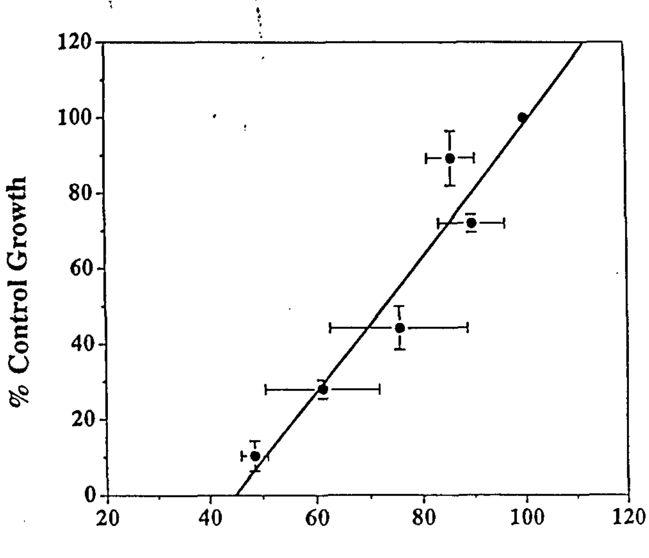


Figure 10. Relationship between growth inhibition and suppression of c-*myc* expression in MCF-7 cells. Data were taken from Figures 1 and 7 respectively.

functional status of the p53 tumour suppressor gene could play a critical role in cell sensitivity to ionizing radiation (Lee and Bernstein, 1993, O'Connor *et al.* 1993, Fan *et al.* 1994, McIlwerth *et al.* 1994, Namba *et al.* 1995, Russell *et al.* 1995, Tsang *et al.* 1995, Zhen *et al.* 1995, Siles *et al.* 1996, Yount *et al.* 1996). However, it is not evident that radiosensitivity is related to the G_1 arrest function of p53—as abrogation of G_1 arrest does not result in a uniform alteration in radiation sensitivity (Jung *et al.* 1992, Brachman *et al.* 1993, Slichmayer *et al.* 1993, Fan *et al.* 1995, Lebreque *et al.* 1995, Gudas *et al.* 1996).

In the present studies, the antiproliferative effects of ionizing radiation were compared in the p53 wild-type MCF-7 breast tumour cell line and in the MDA-MB231 breast tumour cells, where a mutation in p53 abrogates its transactivation function (Bartek *et al.* 1990). The capacity of ionizing radiation to inhibit proliferative capacity after 72 h was not reduced in the MDA-MB231 cells despite the fact that no G_1 arrest was observed in this cell line; in fact, at 0·5 and 1 Gy, the MDA-MB231 cells appeared to be slightly more sensitive to ionizing radiation than the MCF-7 cells. Consequently, abrogation of G_1 arrest by a mutation in p53 does not appear to produce a concomitant decrease in the sensitivity to ionizing radiation, at least in these two non-isogenic experimental breast tumour cell lines. Nevertheless, a similar conclusion was reached by Gudas *et al.* (1996) in studies of MCF-7 breast tumour cell lines selected for resistance to various chemotherapeutic agents.

In addition to G_1 arrest, a p53-dependent factor which should influence radiation sensitivity is the capacity of the cell to undergo apoptotic cell death. Ionizing radiation has been shown to induce apoptosis in various experimental tumour cell lines (Warters *et al.* 1992, Radford *et al.* 1994, Seki *et al.* 1994, Zhan *et al.* 1994, Ling *et al.* 1995, Palayoor *et al.* 1995). However, our analysis of MCF-7 and MDA-MB231 cell lines using both morphological criteria and *in situ* fluorescent end-labelling of DNA demonstrates that neither cell line undergoes cell death, apoptotic or otherwise, at least over the first 96 h after irradiation. The absence of apoptotic cell death as a primary response to irradiation may account for the similar profile of radiation sensitivity in the MCF-7 and MDA-MB231 breast tumour cell lines despite the difference in their p53 status. These findings provide support for the concept that p53 may not influence radiosensitivity unless there is differential induction of apoptotic cell death.

It is, of course possible, and perhaps likely that both MCF-7 and MDA-MB231 cells might eventually demonstrate cell death (apoptotic or otherwise) after prolonged growth arrest. However, this cell death would not reflect the primary response of these cells to irradiation, which is apparently loss of proliferative capacity.

MCF-7 cells apparently have the capacity to engage the apoptotic response pathway in response to certain treatment modalities (Shao *et al.* 1995, Sumantran *et al.* 1995, Texiera *et al.* 1995). However, work from this laboratory (Fornari *et al.* 1994 and unpublished data) as well as reports by other investigators (Oberhammer *et al.* 1993, Zhan *et al.* 1994, Sokolova *et al.* 1995) support the concept that MCF-7 cells are relative refractory to DNA-damage induced apoptosis. For instance, Zhan *et al.* (1994) have reported that apoptosis was observed in MCF-7 cells only after 72 h at an elevated dose (20 Gy) of ionizing radiation, whereas the clinically relevant dose is approximately 10-fold lower. Similarly, Sokolova *et al.* (1995) reported the induction of apoptosis in response to the topoisomerase II inhibitor, VP-16 in MCF-7 cells only after extended exposure to an elevated dose of drug. Recently, Whitacre and Berger (1997) demonstrated that adherent cells are quite refractory to apoptosis; in a panel of 17 cell lines, MCF-7 breast tumour cells showed the lowest incidence of PARP cleavage, an indicator of susceptibility to apoptosis.

As is the case for MCF-7 cells, there *is* evidence for apoptotic cell death in MDA-MB231 cells in response to treatments which do not induce DNA damage (Katayose *et al.* 1995, Perry *et al.* 1995, Seewaldt *et al.* 1995). However, similar to our own conclusions, Siles *et al.* (1996) have reported the absence of apoptosis in response to irradiation. While we cannot readily explain the indication of a sub-G_0 population in irradiated MDA-MB231 cells, we believe this reflects non-specific damage to DNA. Furthermore, a sub-G_0 population may not be an unequivocal indicator of DNA fragmentation associated with the apoptotic pathway.

Because of its role in cell-cycle transition (Seth *et al.* 1993, Karn *et al.* 1989, Shichiri *et al.* 1993) and in the induction of apoptosis (Evan *et al.* 1992, Henneking and Eick, 1994, Packham and Cleveland, 1995), we have been interested in alterations in the expression of c-*myc* in response to agents which induce DNA damage (Gewirtz *et al.* 1993, Bunch *et al.* 1994, Fornari *et al.* 1996), such as the topoisomerase II inhibitors (Osheroff 1989, Chen and Liu, 1994). The current studies demonstrate that ionizing radiation, a modality which induces DNA damage (Whittaker *et al.* 1995), suppresses c-*myc* expression in MCF-7 breast tumour cells. Woloschak and Chang-Liu (1995) reported that ionizing radiation suppresses c-*myc* expression in Syrian hamster embryo cells, but were unable to demonstrate dose-dependent effects as c-*myc* expression was below the limits of detection after exposure to the lowest radiation dose.

A number of studies have reported the long-term *induction* of c-*myc* expression in irradiated cells as a component of radiation-induced transformation and stimulation of tumour cell growth (Sawey *et al.* 1987, Garte *et al.* 1990, St. Clair *et al.* 1991, Mothersill *et al.* 1994). These findings are consistent with a role for c-*myc* in cellular proliferation; however, these observations do not impact upon the current work assessing the *acute* response to radiation induced growth inhibition.

In apparent contrast to our own work, as well as that of Woloschak and Chen Liu (1995), other investigators have reported up-regulation of c-*myc* expression in irradiated cells. For instance, Wilson *et al.* (1993) report an increase in c-*myc* expression and Myc protein levels by ionizing radiation at 15 Gy in primary human B cells, Prasad *et al.* (1995) reported the transient induction of c-*myc* expression in Epstein-Barr-transformed human lymphoblast cells and De Nardo *et al.* (1995) demonstrated a marked increase in c-*myc* expression at 3 and 24 h after irradiation of human breast tumour xenografts in nude mice (at the relatively high dose of 30 Gy). However, these studies did not include MCF-7 or MDA-MB231 cells nor was there an assessment of dose-dependent effects of radiation on c-*myc* expression. Indeed, we found that although c-*myc* expression was modestly altered in MDA-MB231 cells after irradiation, the response was dose-*independent* in these cells. In the absence of evidence for a dose-dependent effects of radiation on c-*myc* expression, the nature of the relationship between up-regulation of c-*myc* expression and either antiproliferative or cytotoxic effects of radiation remains to be established.

In the work reported here, the dose-dependent reduction in c-*myc* expression by ionizing radiation in MCF-7 breast tumour cells and its correspondence with growth inhibition could be related, in part, to the capacity of ionizing radiation to induce G_1 arrest. In contrast, the transient and dose-*independent* suppression of c-*myc* expression in MDA-MB231 cells could be related to the absence of G_1 arrest in this cell line with mutated p53. Nonetheless, despite the differences in c-*myc* expression and p53 status, both cell lines undergo nearly identical inhibition of proliferation—suggesting that in breast tumour cells growth inhibition in response to radiation can occur by pathways which are independent of both p53 and c-*myc*.

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FIRST PROOF

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Report

EB 1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumor cells

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Key words: adriamycin, apoptosis, breast tumor cells, EB 1089, vitamin D

Summary

Exposure of MCF-7 breast tumor cells to the vitamin D_3 analog, EB 1089 enhances the response to adriamycin. Clonogenic survival studies indicate that EB 1089 shifts the dose-response curve for sensitivity to adriamycin by approximately six-fold in p53 wild-type MCF-7 cells; comparative studies in MCF-7 cells with a temperature-sensitive dominant negative p53 mutation show less than a two-fold shift in adriamycin sensitivity in the presence of EB 1089. The combination of EB 1089 with adriamycin also promotes apoptotic cell death in the p53 wild-type MCF-7 cells but not in the MCF-7 cells expressing mutant p53. EB 1089 treatment blocks in increase in $p21^{waf1/cip1}$ levels induced by adriamycin and interferes with induction of MAP kinase activity by ionizing radiation, effects which could be related to the capacity of EB 1089 to promote secretion of insulin-like growth factor binding protein. Taken together with our previous findings that EB 1089 enhances breast tumor cell sensitivity to ionizing radiation, there studies further support the concept that this vitamin D_3 analog could have utility in combination with conventional chemotherapy and/or radiotherapy in the treatment of breast cancer.

Introduction

The anthracycline antibiotic, adriamycin (doxorubicin) is one of the primary chemotherapeutic agents utilized in the treatment of breast cancer [1]. However, the effectiveness of adriamycin, like that of many other antitumor drugs, is limited by its narrow therapeutic index and the consequent severe patient toxicities [2]. Consequently, it would prove advantageous if approaches could be developed for enhancing drug potency without corresponding increases in drug toxicity.

Studies in various tumor cell lines have demonstrated that vitamin D_3 analogues, which are less hypercalcemic than the parent compound [3, 4], can be successfully combined with conventional chemotherapeutic drugs [5–7]. Ravid et al. have recently demonstrated that vitamin D_3 is capable of enhancing the susceptibility of breast tumor cells to adriamycin induced oxidative damage in studies which utilized relatively high concentrations of adriamycin [8]. A recent report from our own laboratory demonstrated that the vitamin D_3 analog EB 1089 enhances radiation sensitivity in MCF-7 breast tumor cells [9]. These studies further indicated that EB 1089 promoted apoptosis in response to ionizing radiation in MCF-7 cells which are generally refractive to apoptotic cell death after irradiation [10]. Finally, this work suggested that functional p53 was necessary for the enhanced response to EB 1089 and radiation.

In the current work, we have studied the effect of EB 1089 on the response of MCF-7 breast tumor cells to clinically relevant concentrations of adriamycin and have initiated studies to investigate the mechanistic basis for the promotion of apoptotic cell death in the breast tumor cell by adriamycin and radiation in the presence of the vitamin D_3 analog.

AUTHOR'S PROOF

Spare set - for your information only, please retain

Materials and methods

Materials

The p53 wild-type MCF-7 human breast tumor cell line was obtained from NCI, Fredrick, MD. MCF-7 cells transfected with a dominant negative temperature-sensitive mutant p53 (p53-143 val-ala) were provided by Dr. Eliot M. Rosen and Dr. Saijun Fan of the Long Island Jewish Medical Center/Albert Einstein College of Medicine. The vitamin D_3 analog, EB 1089 was provided by Dr. Lise Binderup, Leo Pharmaceuticals, Denmark. RPMI 1640 and supplements were obtained from GIBCO Life Technologies, Gaithersburg, MD. Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Boehringer Manheim, Indianapolis, IN. The primary antibodies used for western blotting were purchased from Pharmingen (mouse monoclonal p53); and Transduction Laboratories (mouse monoclonal p21) Horse radish peroxidase conjugated secondary antibodies were obtained from KPL, Gaithersburg, MD. All other reagents used in the study were analytical grade.

Cell culture

All cell lines were grown from frozen stocks in basal RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin at 37°C under a humidified, 5% CO_2 atmosphere.

All experiments were conducted using approximately 10^4 cells per square centimeter at day 0 with the use of time-equivalent and concentration-equivalent controls. The results shown are averages of two to three experiments. Statistical evaluation of quantitative data was performed by analysis of variance with a comparison of multiple means by the Fishers/Scheffe test. Differences with $P < 0.05$ were considered significant. Duplicate measurements were performed on each sample assayed.

Cell viability determination

The effects of adriamycin and EB 1089, alone and in combination on cell viability were evaluated by trypan blue exclusion. For the drug combinations tudies, cells were treated with EB 1089 (100 nM) for 72 h followed by acute exposure (2 h) to adriamycin (1 μM). Cells were harvested using trypsin, stained with 0.4% trypan blue dye and trypan blue negative cells were counted under phase contrast microscopy.

Clonogenic survival

MCF-7 cells were treated with EB 1089 (100 nM) for 72 h followed by acute exposure to varying concentrations of adriamycin (0–100 nM) for a period of 2 h. Cells were trypsinized under sterile conditions immediately following adriamycin treatment and plated in triplicate in 6 well tissue culture dishes at approximately 1,000 cells for each condition (0–10 nM adriamycin) and 3,000 cells for the group treated with 100 nM adriamycin. After 10–14 days, the cells were fixed with 100% methanol, air-dried for 1–2 days and stained with 0.1% crystal violet. For computing the survival fraction, groups of 50 or more cells were counted as colonies and normalized for every 1,000 cells plated.

Cell morphology

At the appropriate intervals after drug treatment, cells were washed and cytocentrifuged onto microscopic slides. The cells were then air dried, stained with Wright-Giemsa stain and photographed under a Nikon light microscope.

TUNEL assay for apoptosis

The method of Gavrielli et al. [11] was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the *In Situ* Cell Death Detection Kit (Boehringer-Manheim). In this assay, the fragmented DNA in individual cells was end labeled using fluorescein dUTP at strand breaks by the enzyme terminal transferase. The slides were then washed, mounted in Vectashield and photographed using a Nikon fluorescent microscope.

Alkaline Unwinding assay for DNA fragmentation

The induction of DNA fragmentation was substantiated using the method of alkaline unwinding, as described previously [9]. Briefly, this involved determination of the ratio of double-stranded and single stranded DNA at 48 h after exposure to adriamycin.

Western blotting

After the indicated drug treatments, cells were centrifuged, washed with PBS and lysed using 100–200 μl of lysis buffer containing protease inhibitors for 30 min in ice. Protein concentrations were determined by the Lowry method and equal aliquots of protein (10 or 20 μg) were separated using 15% SDS PAGE. Proteins were transferred onto a nitrocellulose membrane and blocked in TBS-tween buffer containing 5% non-fat dry milk. Membranes were immunoblotted with respective antibodies at a dilution of 1:5000 (p53); 1:500 (p21); and then incubated with horse radish peroxidase conjugated secondary antibody at 1:2500 (goat anti mouse). Proteins were visualized using enhanced chemiluminescence kit from PIERCE. Equal loading of proteins were confirmed by staining the membrane with Ponseau-S.

MAP kinase activity

At the indicated times after exposure to EB 1089, irradiation or the combination, pelleted cells were washed in PBS and snap frozen. Cell pellets were lysed in lysis buffer containing 5 mM EGTA, and 5 mM EDTA supplemented with protease inhibitors. Lysates were clarified by centrifugation at 5,000 × *g* at 4°C for 5 min. MAP kinase was immunoprecipitated with a primary MAP kinase antibody (mouse monoclonal) followed by a secondary rabbit anti-mouse antibody. Protein A agarose was added to immunoprecipitate the protein-antibody complex. The activity of MAP kinase was assayed as described by Reardon et al. [12] using myelin basic protein as substrate. Preimmune controls were included to ensure selectivity of substrate phosphorylation. The reaction was terminated by transfer to p81 filter paper; filters were rinsed repeatedly in 185 mM orthophosphoric acid and then dehydrated in 100% ethanol. Total radioactivity on filters was determined by liquid scintillometry.

Results

Influence of EB 1089 on the response of MCF-7 cells to adriamycin

Figure 1 shows that prior exposure to EB 1089 enhanced the response of MCF-7 cells to adriamycin. While EB 1089 (100 nM) and adriamycin (1 μM) each alone reduced cell growth by ~50% and 70%, respectively, combined treatment of cells with EB 1089 followed by adriamycin resulted in an approximately 90% reduction in final cell number compared to growth of untreated controls.

The concentration of adriamycin utilized for the studies presented in Figure 1 (1 μM) reflects the peak concentration range achieved in the clinic after a pulse exposure to drug [13]. This concentration of adriamycin produces a greater than 1 log dose reduction in clonogenic survival (data not shown). In order to discern the influence of EB 1089 on clonogenic sensitivity to adriamycin, studies were performed over a range of adriamycin concentrations (1–100 nM) where the effects of adriamycin alone on clonogenic survival would be less pronounced. Figure 2A indicates that

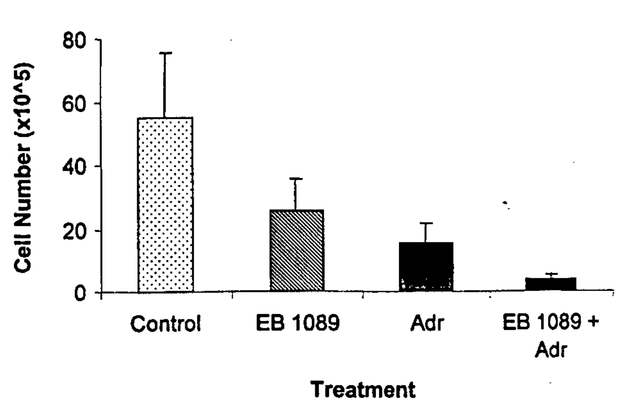

Figure 1. Influence of pretreatment with EB 1089 (100 nM) on the antiproliferative effects of adriamycin in human breast tumor cells (MCF-7). Cells were treated with EB 1089 for 72 h and replenished with fresh media prior to adriamycin treatment (1 μM for 2 h). Cells were then allowed to grow at 37°C for an additional 48 h before assessing viable cell number. Data presented are means ± SEM of two experiments.

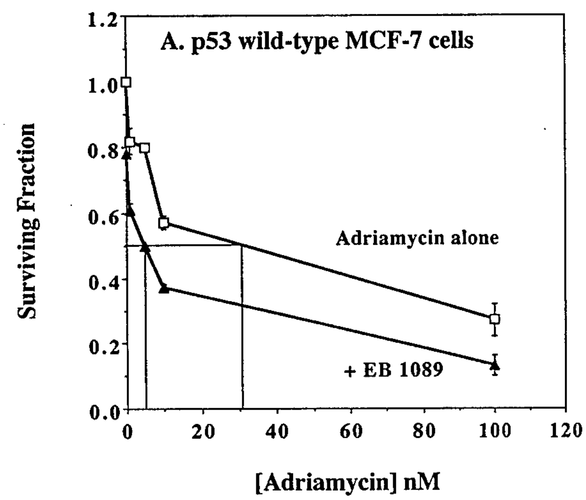

Figure 2. Clonal survival of MCF-7 wild-type and MCF-7 cells with a dominant negative p53 mutant gene after exposure to varying concentrations of adriamycin (0–100 nM) for 2 h with or without EB 1089 pretreatment (48 h). Data represent means ± SEM of two independent experiments.

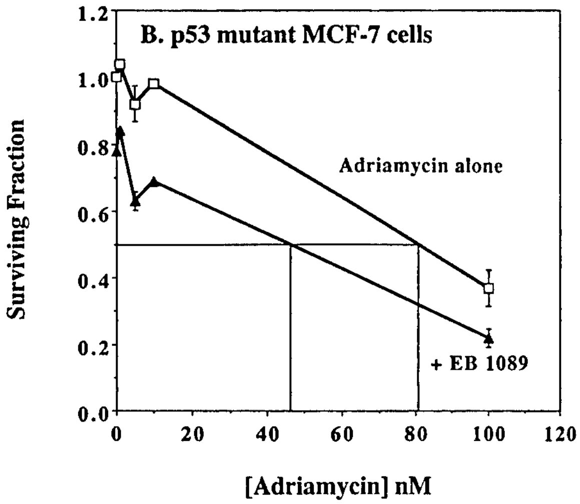

Figure 2. (*continued*)

the IC_{50} value for loss of clonogenic survival was approximately 30 nM in the MCF-7 cells; EB 1089 induced a shift in the dose response curve such that the concentration of adriamycin required to produce a 50% reduction in clonogenicity was reduced six-fold (to approximately 5 nM).

Role of p53 in the potentiation of the response of the breast tumor cell to adriamycin

An additional series of studies were performed in order to determine whether the p53 status of the breast tumor cells would affect the influence of EB 1089 on the response to adriamycin. Using an MCF-7 cell line with a dominant negative temperature sensitive mutant p53 at the permissive temperature of 37°C [14], the IC_{50} for adriamycin was determined to be approximately 80 nM (Figure 2B). This observations is, of itself, quite interesting in that wild-type p53 function appears to promote adriamycin sensitivity, as suggested by earlier work [15, 16]. Figure 2B further indicates that pretreatment with EB 1089 induced a shift in the dose response curve such that the concentration of adriamycin required to produce a 50% reduction in clonogenicity was reduced less than two-fold (to approximately 45 nM) in the p53 mutant cells.

Indications of apoptotic cell death after combined treatment with EB 1089 and adriamycin

We have previously reported that MCF-7 cells fail to undergo apoptotis in response to clinically relevant doses of adriamycin [17, 18] and even after supraclinical doses of radiation [10], and that EB 1089 promotes apoptosis in the irradiated cells [9]. We therefore monitored the capacity of EB 1089 to promote apoptosis in response to adriamycin based on cell morphology as well as DNA fragmentation by TUNEL analysis.

Figure 3 indicates that neither EB 1089 nor adriamycin alone produced a significant degree of apoptosis in either the p53 wild-type or the p53 mutant MCF-7 cells; although a few apoptotic cells could be identified in each field, the absence of a significant cell population demonstrating shrinkage, nuclear condensation and apoptotic body formation is consistent with the general refractoriness of these cells to apoptotic cell death [19–21]. In fact, the cells tended to demonstrate an increase in size, as reported previously [17, 18]. Exposure of the p53 wild-type MCF-7 cells to EB 1089 prior to their being challenged with adriamycin provided unequivocal morphological evidence of apoptotic cell death. Conversely, in p53 mutant cells, prior exposure to EB 1089 failed to promote apoptosis in response to adriamycin.

The morphological findings presented in Figure 3 are supported by the DNA fragmentation data generated using the TUNEL assay shown in Figure 4. In terms of the p53 wild-type MCF-7 cells, while a few fluorescent cells were evident with adriamycin alone, the fluorescent cell population was clearly increased by the combination of EB 1089 with adriamycin (Figure 4). With regard to the p53 mutant cells, some generalized fluorescence was detected in controls; [we believe that this may indicate increased sensitivity to spontaneous DNA damage, reflecting the p53 status of this cell line.] However, the extent of fluorescence was not increased by EB 1089 alone, adriamycin alone or the combination of EB 1089 with adriamycin (Figure 4).

The promotion of DNA fragmentation in the MCF-7 cells by the combination of EB 1089 with adriamycin was substantiated using alkaline unwinding. In two experiments, EB 1089 increased the extent of DNA breakage (over baseline levels) by 80% and 100%, respectively (not shown).

Potential role of p21waf1/cip1 in the promotion of apoptosis by adriamycin in the presence of EB 1089

The studies described above (as well as our previous work combining EB 1089 with irradiation) indicated that EB 1089 has a permissive effect on the promotion of apoptosis in the breast tumor cell. In order to investigate the mechanistic basis for the

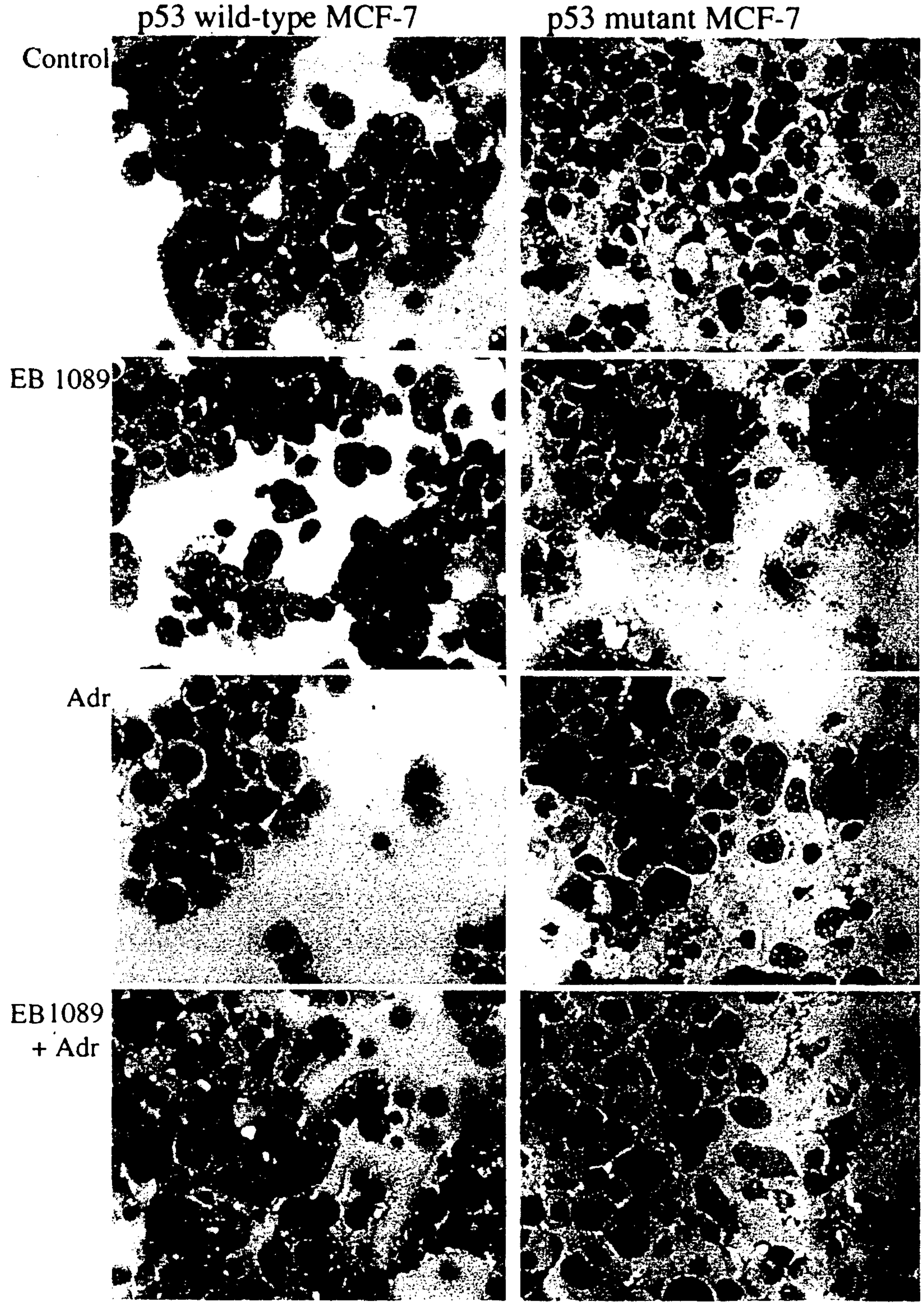

Figure 3. Effects of EB 1089, adriamycin and the combination of EB 1089 with adriamycin on morphology of MCF-7 (wild-type and p53 mutant) breast tumor cells as determined by light microscopy. Cells were exposed to EB 1089 followed by adriamycin treatment (as described in the legend for Figure 1) and allowed to grow 48 h before processing for morphological analysis.

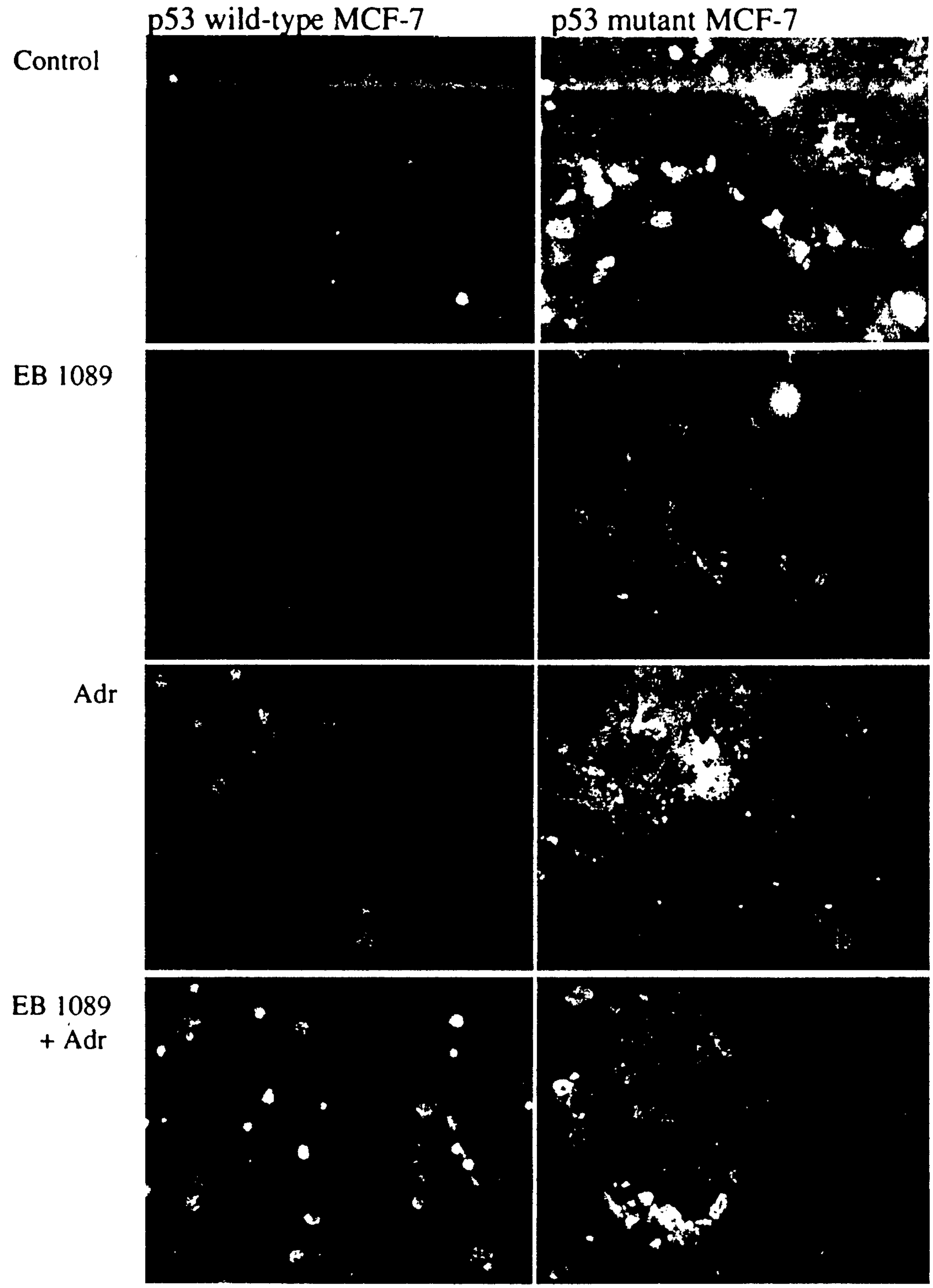

Figure 4. Effects of combining EB 1089 with adriamycin (1 μM) on the induction of DNA fragmentation in MCF-7 (wild-type and p53 mutant) cells as determined by fluorescent end labeling. Cells were isolated 48 h following adriamycin treatment, cytospun onto glass slides and stained according to the TUNEL protocol as described in the methods section.

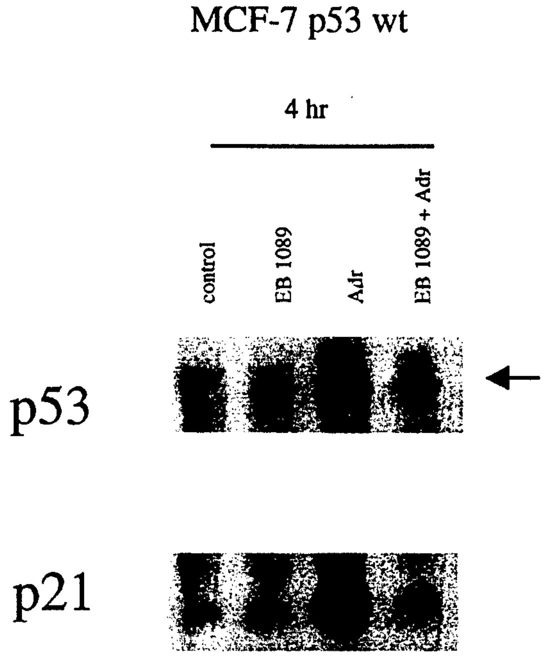

Figure 5. Representative western blot analysis of p53 and p21 expression in MCF-7 wild-type cells in response to treatment with EB 1089 and adriamycin alone and in combination.

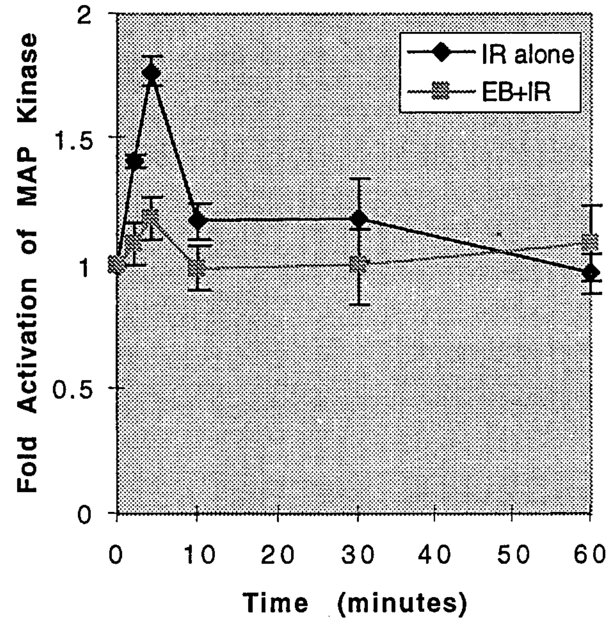

Figure 6. Comparison of MAPK activation in MCF-7 wild-type cells in response to ionizing radiation (10 Gy) and the combination of EB 1089 with irradiation at the indicated time intervals. Data represents the mean ± standard error of three independent experiments.

promotion of apoptosis by adriamycin and irradiation in the presence of EB 1089, we considered the possibility that abrogation of the adriamycin induced increase in p21$^{waf1/cip1}$ levels might lead to preferential expression of the apoptotic function of p53 in the MCF-7 cells [22–24]. The p53-mediated response to adriamycin was investigated by measuring p53 protein expression while the transcriptional activity of p53 was assessed based on the increase in p21$^{waf1/cip1}$ protein levels. Western blot analyses presented in Figure 5 indicates that the levels of the tumor suppressor protein p53 and the cyclin dependent kinase inhibitory protein p21$^{waf1/cip1}$ were increased in response to adriamycin alone. However, the adriamycin-induced increase in p53 was partially suppressed while the adriamycin-induced increase in p21$^{waf1/cip1}$ levels was essentially abrogated when EB 1089 preceded exposure to adriamycin.

Potential role of MAP kinase in the promotion of apoptosis by adriamycin in the presence of EB 1089

Previous studies from this laboratory have demonstrated that EB 1089 enhances the response to ionizing radiation in p53 wild-type cells [9], similar to the current findings relating to adriamycin. Ionizing radiation has been demonstrated to increase MAP kinase activity in the breast tumor cells [12]; furthermore p21$^{waf1/cip1}$ expression is potentially linked to MAP Kinase activity [25–27]. Consequently, we investigated whether EB 1089 had the capacity to interfere with activation of MAP kinase. Figure 6 indicates that 10 Gy of radiation produced a transient stimulation of MAP Kinase activity which was virtually eliminated by prior exposure to EB 1089. EB 1089 alone had no effect on MAP kinase activity (not shown).

Discussion

The findings presented in this paper build upon our own recent work as well as studies by other investigators which support the concept that vitamin D_3 and/or its analogs have potential utility in combination with conventional chemotherapeutic agents in the treatment of various malignancies. Reports by Cho et al. [6], Light et al. [7] and Moffatt et al. [28] have demonstrated that vitamin D_3 or its analogs are effective in combination with platinum drugs against prostate tumor, squamous carcinoma and breast tumor cells. Vitamin D_3 and its analogs have also been utilized in combination with TNF, tamoxifen, paclitaxel, ionizing radiation, and adriamycin in studies involving breast tumor cells [5, 8, 9, 29, 30].

Our present work extends the current literature by evaluating the capacity of EB 1089, a vitamin D_3 analog with limited toxicity [30–33], to promote apoptosis in response to a clinically relevant concentrations of adriamycin [34], and determining the potential involvement of p53 function in the apoptotic response. The data presented in this report indicate that the combination of EB 1089 with adriamycin has the potential to significantly interfere with tumor cell growth – based on a reduction in viable cell number as well as a shift in the dose response curve for sensitivity to adriamycin of MCF-7 cells. The difference in the magnitude of the shift in the dose response curve (six-fold for the p53 wild-type cells and less than two-fold shift for the p53 mutant cells) suggests that p53 status (and, by inference, p53-dependent apoptosis) could be factors in potentiation of sensitivity to adriamycin, as reported previously for the combination of EB 1089 with radiation [9]. Why this would occur is not immediately evident since the promotion of apoptosis does not appear to directly affect clonogenic cell survival. That is, the curves for clonogenic survival in the absence and presence of EB 1089 are parallel and virtually superimposable (for both the p53 wild-type and the p53 mutant MCF-7 cells) indicating that the shift in the dose response curve observed in the presence of EB 1089 is solely a function of the additive antiproliferative and cytotoxic effects of EB 1089 and adriamycin. These observations are consistent with earlier reports suggesting that the induction of apoptosis can accelerate cell death without influencing ultimate cell survival [35, 36].

The basis for the relative refractoriness of breast tumor cells to DNA damage induced apoptosis as well as for the permissive effects of EB 1089 on the promotion of apoptosis in response to adriamycin or irradiation remain to be fully elucidated. While the absence of caspsase 3 in MCF-7 cells [37] may limit the capacity of these cells to undergo a full-fledged apoptotic response [19, 38, 39], apoptotic cell death has been reported in response to a variety of (non-DNA damaging) drugs including retinoids, tamoxifen and taxol [40–42] suggesting that refractoriness to apoptosis in the breast tumor cell may occur primarily in response to agents which induce DNA damage.

MAP kinase as well as P13 kinase and Akt kinase activities have been linked to the regulation of cell survival and cell death through the axis involving insulin like growth factors and insulin like growth factor binding proteins and the phosphorylation/inactivation of the pro-apoptotic BAD protein [41–46]. The fact that EB 1089 blocks activation of MAP kinase while promoting apoptosis in response to adriamycin and ionizing radiation suggests that the effects of EB 1089 may mediated through the secretion of the pro-apoptotic insulin-like growth factor binding proteins [47]. Studies to determine the potential interaction(s) between EB 1089 and insulin like growth factor are currently in progress.

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FIRST PROOF

Report

Growth arrest and cell death in the breast tumor cell in response to ionizing radiation and chemotherapeutic agents which induce DNA damage

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Key words: adriamycin, apoptosis, DNA damage, growth arrest, ionizing radiation

Summary

Introduction

The current emphasis on apoptotic cell death as the tumor cell response to chemotherapeutic drugs and ionizing radiation trends to overshadow the potential contribution of alternative responses which also facilitate loss of proliferative capacity and/or cell death. These include prolonged growth arrest associated with replicative senescence [1–3], prolonged growth arrest succeeded by reproductive cell death (mitotic catastrophe) [4–6] as well as transient growth arrest followed subsequently by apoptosis or necrosis. Response pathways which are independent of apoptosis may be particularly relevant to solid tumors such as breast cancer, where a primary apoptotic response to agents which induce DNA damage appears to be the exception rather than the rule [7]. This commentary will be focused on the apoptotic and non-apoptotic responses of the breast tumor cell to therapeutic modalities which induce DNA damage, specifically ionizing radiation and chemotherapeutic drugs which are inhibitors of topoisomerase I and II (emphasizing the response to adriamycin).

Elements of the apoptotic pathway

The basic elements of the apoptotic response to DNA damage have been well characterized [8, 9], and consequently only a brief overview is presented in this commentary. The pathway leading to induction of apoptosis in response to DNA damage is generally thought to be initiated at the level of p53, since apoptotic function is frequently compromised or abrogated in cells with mutated or nonfunctional p53 [10, 11] furthermore, mutations in p53 are frequently associated with reduced chemosensitivity and radiosensitivity [12–15] although this is by no means a universal finding, particularly with regards to radiation [16–19]. Functional p53 is not an absolute requirement for the promotion of apoptosis since both p53 null and p53 mutated cells have been shown to undergo apoptosis quite rapidly and effectively [20–24].

DNA damage induces stabilization of p53 [25, 26]; the induction of p53 is mediated, in part, by the activity of the ATM protein upstream of p53 [27, 28] and further facilitated by the p14 ARF proteins which block p53 degradation [29, 30]. Overexpression of either Myc or E2F-1 contribute to the apoptotic response [31–34] through inhibition of mdm2 mediated degradation of p53 [35, 36]. p53 is thought to directly influence the levels of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 proteins [37]. The ratio of the Bax and Bcl-2 proteins [38, 39] as well as the phosphorylation mediated activation/inactivation of Bcl-2 [40, 41] appear to be critical to the regulation of the DNA damage induced apoptosis; however, levels of other members of these protein families including Bad and Bclx are also likely to be determinants of the propensity of the cell to mount an apoptotic

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Spare set - for your information only, please retain response [42–45]. A favorable ratio of pro-apoptotic to anti-apoptotic family proteins induces a reduction in mitochondrial membrane potential and/or perturbations in the mitochondrial permeability transition pore complex [46, 47], resulting in the release of cytochrome c [48]; in turn, cytochrome c release regulates activity of the family of caspases [49, 50]. Cytochrome c binding to apoptotic protein activating factor (APAF) activates executioner caspases, including caspases 9 and 3 [51–53] while the final events of genomic degradation into nucleosomal fragments are thought to occur through the proteolytic activity of caspases subsequent to the cleavage of DNA fragmentation factor [54].

Growth arrest and cell death in response to DNA damage in the breast tumor cell

Tumor cells of hematopoietic or lymphatic origin and various solid tumor cell lines frequently undergo apoptotic cell death after irradiation or exposure to adriamycin [9, 18, 19, 22, 23, 56–61]. In contrast, breast tumor cells tend to be relatively refractory to apoptosis in response to either irradiation or exposure to chemotherapeutic drugs which induce DNA damage [62–70]. That is, while both ionizing radiation and adriamycin effectively abrogate clonogenic survival of the breast tumor cell [71, 72], the primary response to these modalities is prolonged growth arrest [66, 69]. We have previously reported that irradiation results in an extended period of growth arrest in both p53 wild-type MCF-7 and p53 mutated MDA-MB231 breast tumor cells [69]. Strobl et al. [73] also recently reported mitotic arrest and giant cell formation in irradiated MCF-7 cells. We have further determined that while the initial response after either acute exposure to 1 μM adriamycin or chronic exposure to 50 nM adriamycin is non-apoptotic cell death of between 20–35% of the cell population, the subsequent response to adriamycin is prolonged growth arrest [63, 66][1]. Roninson's group has recently presented findings indicating that chronic exposure of breast tumor cells to low dose adriamycin produces signs of replicative senescence [3, 74].

Although neither ionizing radiation nor adriamycin induce a primary apoptotic response in the breast tumor cell, apoptotic cell death has been reported to occur in the breast tumor cell in response to prolonged drug exposure and/or elevated drug concentrations. Leung et al. [75] indicated that continuous exposure for 24 h to 1 or 5 μM adriamycin promoted apoptosis in MCF-7 cells while Ruiz-Ruiz et al. [76] reported apoptosis after continuous (48 h) exposure of both MCF-7 and EVSA-T cells to (1 μM) adriamycin. Andres et al. [77] found that while apoptosis failed to occur (and cell number was not reduced) in MCF-7 cells exposed (for 1 h) to adriamycin up to a concentration of 5 μM, p53 mutated MDA-MB453 breast tumor cells underwent apoptosis under the same conditions. Similarly, Fan et al. [78] demonstrated that exposure to 10 μM adriamycin for 1 h produced DNA fragmentation in the p53 mutated MDA-MB231 cell line. Both Hansen et al. [79] and Koutsileris et al. [80] reported DNA laddering after 48 h of continuous exposure of MDA-MB231 cells to a relatively low adriamycin concentrations (0.1 μM). Taken together, these findings support the concept that p53 mutant breast tumor cells may be more susceptible to apoptosis than p53 wild-type cells and are consistent with recent reports by Bunz et al. [81, 82] suggesting preferential induction of apoptosis in p53 mutated cells.

Like adriamycin, the epipodophyllotoxins, VM-26 (teniposide) and VP-16 (etoposide) are topoisomerase II inhibitors [83] to which the breast tumor cells are relatively resistant in terms of an apoptotic response. Benjamin et al. [84] were able to demonstrate caspase activation and PARP cleavage with exposure of MCF-7 cells to 100 μM VP-16 for between 6 and 16 h; however, the apoptotic response was facilitated by prior serum starvation. Wilson [85] reported that no apoptosis was evident in MCF-7 cells after exposure to up to 50 μM VP-16, Sumantran et al. [86] failed to detect apoptosis by 2 μM VP-16 in MCF-7 cells even 6 days after drug exposure while Leung et al. [75] reported that continuous exposure for 24 h to 1–5 μM VP-16 failed to induce apoptosis in MCF-7 cells. Likewise, Gibson et al. [87] reported that exposure of MCF-7 or MDA-MB435 cells to VP-16 (even at 100 μM) for as long as 72 h had no detectable effect on viable cell number and did not produce sub Go (i.e. apoptotic) cells; only growth arrest was evident. In support of the idea that prolonged drug exposure is required to induce an apoptotic response, Sokolova et al. [88] demonstrated DNA laddering in MCF-7 cells after 8 days of exposure to 50 μM VP-16.[2]

The capacity of camptothecin, an inhibitor of topoisomerase I, to induce apoptosis in breast tumor cells is also of interest, as camptothecin has been proposed to induce double-strand DNA breaks through collision of the advancing replication fork with the topoisomerase I-DNA complex [89]. In studies by

Weurzberher et al. [90], a 1-μM pulse of camptothecin failed to induce apoptosis in MCF-7 cells even 6 days post-treatment and the response to this drug appeared to be exclusively growth arrest. In fact, even supralethal concentrations of camptothecin resulted in apoptosis in only 25% of the cell population; in contrast, beta lapachone induced a significant degree of apoptosis via a p53-independent pathway [90]. In support of these findings, studies in the NCI panel of tumor cell lines found that resistance to apoptosis in response to camptothecin was evident in both MCF-7 and T47D breast tumor cells [91]; these investigators furthermore indicated that apoptosis failed to correlate with growth inhibition, strongly suggesting that other mechanisms of cell death were involved in the toxicity of camptothecin. Leung et al. [75] reported that continuous (24 h) exposure of MCF-7 cells to 5 μM camptothecin did indeed promote apoptosis while Liu et al. [92] found that a 48-h treatment of near-confluent MCF-7 and MDA-MB468 cells with 0.5 μM camptothecin provided clear evidence of DNA fragmentation. Similarly, apoptosis was evident in human breast tumor cell xenografts after 7 days of camptothecin treatment [92]. Recent studies by del Bino et al. [93] also support the capacity of camptothecin to induce apoptosis in MCF-7 cells over a period of 72 h, although this work used a relatively low concentration (0.15 μM) of the drug.

With some exceptions, it appears that elevated drug concentrations, prolonged times of drug exposure or both are required to elicit an apoptotic response to adriamycin, the epipodophyllotoxins or camptothecin in the breast tumor cell, strongly supporting the premise that the apoptotic pathway in the breast tumor cell is intact, but is relatively unresponsive to DNA damage. The concept that breast tumor cells are relatively refractory to DNA damage induced apoptosis is supported by the work of a number of investigators [62, 70, 93, 94]. It is further of interest that adriamycin was found to be incapable of promoting apoptosis in MCF-10A cells, a breast epithelial cell line [95]. Our own recent work indicates that apoptosis in response to either adriamycin or radiation can be facilitated by prior exposure to vitamin D_3 analogs [71, 72] while as described below, the breast tumor cell is quite susceptible to apoptosis in response to a variety of agents which act by pathways which are not associated with the induction of DNA damage. Consequently, it is possible that the apoptotic response to DNA damage (but not other apoptotic signaling pathways) is attenuated in the breast tumor cell.

Factors that may block the apoptotic response to DNA damage in the breast tumor cell

As indicated above, the pathway leading to apoptosis is quite complex, and it is unlikely that the general refractoriness of the breast tumor cell to DNA-damage induced apoptosis can be ascribed to a single element. In fact, multiple regulatory elements may contribute to the attenuation of the apoptotic response of the breast tumor cell to DNA damage. Elements which are likely to have a primary role in the refractoriness of breast tumor cells to apoptosis in response to DNA damage are discussed in the following section. However, the precise role of these regulatory elements in modulating the apoptotic response of the breast tumor cell to DNA damage remains to be defined.

A. p53 mediated apoptosis and elements downstream of p53

p53 and p21$^{waf1/cip1}$

As described in some detail above, p53 gene function is generally considered to be a necessary component of the apoptotic response. Although Takahashi et al. [96] have suggested that p53 in MCF-7 cells is dysfunctional, at least the transactivational function of p53 appears to be intact in MCF-7 cells as DNA damage induces a profound increase in levels of p21$^{waf1/cip1}$ [97–99]. Furthermore, as indicated above, DNA damage induced apoptosis is not limited exclusively to cells with functional p53. The fact that both ionizing radiation and adriamycin fail to induce a primary apoptotic response in both the p53 wild-type MCF-7, and the p53 mutated MDA-MB231 breast tumor cells [66, 69][1] suggests that the functional status of p53 may not be a determinant of the primary apoptotic response of breast tumor cells to DNA damage. Furthermore, there is accumulating evidence that p53 mutated (and/or p21 defective) tumor cells may be *more susceptible* than the p53 wild type cells to a delayed form of apoptosis which reflect a mitotic catastrophe subsequent to growth arrest [80, 81, 100, 101]. That is, the G1 checkpoint function of p53 may protect breast tumor cells from apoptosis by permitting sustained growth arrest in G1, while the G2 arrest function of p53 provides for sustained arrest in G2 [81, 100, 101], whereas transient G2 arrest in p53 mutant cells may lead to delayed apoptotic cell death.

As indicated in the preceding section, a blockade to growth arrest may indirectly influence the apoptotic response since the growth arrest and cell death pathways which respond to DNA damaging agents appear to be closely intertwined at the level of p53 function. In cells with a functional p53 tumor suppressor gene, a post-translational increase in levels of p53 [25, 26], a consequent increase in levels of the cyclin-dependent kinase inhibitory protein $p21^{waf1/cip1}$ [25, 26, 97–99] and the resulting blockade to phosphorylation of Rb family of proteins (which may include Rb, p130, and p107) promotes binding of the Rb proteins to the E2F family of transcription factors [102–105]. Binding of the Rb proteins to E2F converts E2F from a transcriptional activator to a transcriptional repressor of genes which regulate DNA synthesis such as DNA polymerase alpha, thymidine kinase, thymidine synthetase, *c-myc*, and dihydrofolate reductase [106–109] – resulting in a blockade of the G1 to S transition. Recent experimental evidence indicates that abrogation of the $p21^{waf1/cip1}$ component of growth arrest is permissive for the apoptotic functions of p53 [24, 110–113]; consequently, it appears possible that upregulation of $p21^{waf1/cip1}$ by ionizing radiation or agents such as adriamycin may be antagonistic to the apoptotic response in p53 wild-type breast tumor cells. Why this should occur preferentially in the breast tumor cell is not clear at the present time.

Bax and Bcl-2 levels and activity

In terms of the Bax and Bcl-2 families of proteins, both the basal levels of these proteins as well as the regulation of these proteins in response to DNA damage must be considered in evaluating the basis for the lack of a primary response to DNA damage. Levels of the anti-apoptotic Bcl-2 and Bcl-xl proteins are relatively high in MCF-7 cells [114, 115]. Srivistava et al. [116] have reported that DNA damaging agents (in contrast to vinca alkaloids) fail to promote dephosphorylation (and presumably inactivation) of Bcl-2 in MCF-7 and MDA-MB231 breast tumor cells. However, Leung et al. [75] have suggested that apoptosis in response to adriamycin or camptothecin in MCF-7 cells does not appear to be directly related to Bax and Bcl-2 levels, since no apoptosis was observed in response to by VP-16 despite upregulation of Bax. While it has also been reported that overexpression of exogenous Bax or Bcl-xs can sensitize breast tumor cells to various DNA damaging agents [86, 117], such studies do not shed direct light on the role of the endogenous proteins in the regulation of the apoptotic response.

Caspase 3

The failure of MCF-7 breast tumor cells to undergo DNA-damage induced apoptosis is thought to be related, at least in part, to the absence of one of the 'executioner' caspases, caspase 3 [118]. However, caspase 3 is expressed at high levels in MDA-MB231 breast tumor cell lines [115] which also fail to undergo apoptosis in response to ionizing radiation.[1] The fact that MCF-7 cells undergo apoptosis in response to certain non-DNA damaging agents (see below), to DNA damaging agents in the presence of vitamin D_3 analogs [71, 72] or MAP kinase inhibitors (see below) and after prolonged exposure or high concentrations of DNA damaging agents argues against the absence of caspase 3 being a critical factor in abrogation of the primary apoptotic response. In this context, a recent report describes caspase-independent apoptosis in response to vitamin D_3 in MCF-7 cells [119]. Finally, since DNA laddering as well as other indicators of apoptotic cell death have been demonstrated to occur in the MCF-7 cells in response to agents which fail to induce DNA damage, caspase 3 *independent* apoptotic pathways can be readily invoked for this breast tumor cell line.

B. Modulation of the apoptotic response to DNA damage

Activation of NFKappa B

There is accumulating evidence that activation of NF-kappa B blocks the apoptotic response in a variety of cells, including breast cancer [120]. Antineoplastic drugs which induce DNA damage have been shown to activate NF kappa B in the breast tumor cell [121]. Activation of NF-kappa B and the translocation of NF-kappa B to the nucleus occurs through the dissociation of IKB alpha (an NF kappa B inhibitory protein) and its subsequent degradation [122–124]. The blockade to apoptosis through activation of NF-kappa B is thought to occur through the regulation of a spectrum of anti-apoptotic proteins which interfere with caspase activity [125, 126]. Prevention of apoptosis through the activation of NF kappa B has also been implicated in breast carcinogenesis – presumably by preventing cell death which would otherwise occur in cells incurring mutations which might activate select oncogenes or inactivate select tumor suppressor genes [127]. Finally, a linkage between Bcl-2 and NF-kappa B has been noted in that Bcl-2 appears to promote the activation of NF-kappa B by influencing the degradation of IKB alpha [128].

The insulin like growth factor/insulin like growth factor binding protein interaction

An extensive body of evidence indicates that insulin-like growth factor (IGF) and insulin-like growth factor binding proteins (IGFBP) have opposing effects on breast tumor cell survival and the capacity of the breast tumor cell to undergo apoptotic cell death [129]. The IGFB proteins are known to act, in part by binding to and inhibiting the proliferative function and anti-apoptotic functions of IGF as well as through their binding to cell surface receptors [130, 131]. The two arms of the IGF and IGFBP signaling pathways involve, respectively, the MAP kinases [132] and the PI3 and Akt kinases [133–135]. Both arms appear to regulate the phosphorylation and hence inactivation of the pro-apoptotic BAD protein [136, 137]. Drugs which has antiproliferative and cytotoxic activity against the breast tumor cell such as tamoxifen and retinoic acid have been demonstrated to alter the ratio of these secreted proteins to favor the apoptosis-promoting IGFBPs [138, 139].

The fact that vitamin D_3 analogs have been shown to promote secretion of IGFBP from the breast tumor cell [140], that IGFBPs facilitate apoptosis [131, 141], that IGF-1 has been shown to block apoptosis [142–145] taken together with our findings that vitamin D_3 analogs enhance the apoptotic response to adriamycin and radiation in the breast tumor cell [71, 72] supports the hypothesis that the relative levels of these proteins may regulation susceptibility of the breast tumor cells to apoptosis. That is, it is possible that high levels of IGF in the medium of proliferating breast tumor cells represent an additional factor which is antagonistic to the apoptotic response to DNA damage. Why these factors would fail to block apoptotic signaling through non-DNA damage dependent pathways remains an open question, unless DNA damage of itself influences an autocrine response involving insulin like growth factor and its associated binding proteins in the breast tumor cell.

Activation of MAP kinases

Studies have shown that the relative outputs of the stress activated JNK pathway and the cytoprotective MAP kinase (ERK or extracellular signal regulated kinase) pathway determine whether cells live or die in response to environmental insult [146]. Moreover, blockade of the MAP kinase pathway (e.g. by pharmacological inhibitors such as PD 98059) have been shown to promote tumor cell death in response to certain DNA damaging agents [147]. In this context, inhibition of ERK kinase activity has been associated with radiosensitization in the breast tumor cell [148, 149] while recent work has indicated that activation of MAP kinases causes resistance to growth inhibition by IGFBP-3 [132]. Recent reports indicate that MAP kinase and Akt promote phosphorylation of different residues on the BAD protein, serine 136 by Akt kinase and ser 112 by MAP kinase [136, 137]. Consequently, activation of the MAP kinase pathway and PI3 kinase pathways may converge to block apoptosis when IGF is the predominant species in the cell environment.

C. Membrane-mediated effectors of the apoptotic response

Ceramide activation

Studies in a number of laboratories using a variety of experimental tumor cell lines have implicated membrane-associated ceramide generation as having a central role in mediating DNA damage induced cytotoxicity and apoptosis [60, 61, 150, 151]. In fact, resistance to radiation-induced apoptosis has been associated with the absence of ceramide generation [152, 153]. Liu et al. [154] make a convincing case for ceramide degradation conferring resistance to a variety of chemotherapeutic agent in MCF-7 breast tumor cells. Furthermore, treatment of MCF-7 cells with adriamycin was shown to increase ceramide generation and to produce oligosomal fragmentation whereas ceramide was not generated in the drug-resistant cells [155]. However, as noted by the authors [155], ceramide generation required elevated concentrations of adriamycin and prolonged times of drug exposure. Consequently, while a defect in the capacity of the breast tumor cell to generate ceramide could play a role in its refractoriness to DNA damage induced apoptosis, it is not yet clear whether generation of ceramide is an obligatory intermediary step in this pathway.

The CD95 (APO-1/Fas) signaling pathway

Another membrane-associated mechanism which has been strongly identified with apoptosis in a variety of tumor cells involves the CD95 (APO-1/Fas) signaling pathway [156]. This involvement of the CD95 (APO-1/Fas) signaling pathway in apoptosis in response to a variety of DNA damaging agents (including doxorubicin, cytarabine, methotrexate, cisplatin, and etoposide) has been shown most clearly in leukemic cells and in neuroblastoma [157, 158] while a Fas dependent component of 5-FU toxicity has also been identified in colon carcinoma cells [159]. Drug resistance has been found to be associated with deficient activation of the CD95 system in myeloma and leukemia cells [160] while Fulda et al. reported strong induction of CD95 in chemosensitive cells and weak induction in drug resistant cells such as breast tumors [161]. A somewhat indirect relationship between drugs which induce DNA damage and CD95 (APO-1/Fas) signaling pathway has been established in that these agents have been shown to sensitize various tumor cells to Fas mediated apoptosis and toxicity [162–165]. In terms of the breast tumor cell, vitamin E has been reported to induce Fas-mediated apoptosis [166]; however, a recent report strongly suggests that the CD95 (APO-1/Fas) pathway is not implicated in drug-induced apoptosis in the breast tumor cell – as induction of apoptosis and expression of the CD95 ligand in response to genotoxic drugs could be dissociated [76]. This may be due, in part, to the relatively low Fas expression in many breast tumor cell lines (with T-47D cells being a notable exception) [167]. Furthermore, as ceramide has been implicated in mediating doxorubicin and radiation-mediated activation of Fas signaling to apoptosis [168], this pathway may not play a significant role in breast tumor cell signaling by virtue of limited ceramide generation in response to DNA damage.

The role of apoptosis in the response of the breast tumor cell to DNA damage

There is extensive evidence that breast tumor cell lines, even MCF-7 cells, do demonstrate apoptotic cell death in response to a variety of stimuli which are not associated with the induction of DNA damage; these include epidermal growth factor, retinoic acid, tamoxifen, okadaic acid, genistein, and taxol [169–175]. In addition, apoptosis occurs in response to adriamycin and irradiation in the presence of vitamin D_3 analogs [71, 72] and can be enhanced by inhibition of MAP kinase [146, 147]. These data suggest that the signaling pathway leading to apoptotic cell death may be selectively obstructed in breast tumor cells exposed to clinically relevant doses of adriamycin or irradiation and that pharmacologic manipulation of this pathway could be permissive for apoptotic cell death in the breast tumor cell.

The absence of a significant primary apoptotic response in breast tumor cells exposed to adriamycin or irradiation obviously does not reflect therapeutic ineffectiveness since radiation and drugs such as adriamycin are highly effective clinical modalities in the treatment of breast cancer [176]. Nevertheless, as discussed above, there is compelling evidence in the literature that the absence of an apoptotic response may compromise tumor cell sensitivity to drugs and radiation. Previous studies have demonstrated a relationship between lack of responsiveness to chemotherapy (in terms of patient survival and relapse) associated with mutations in p53 [177, 178]. Furthermore, recent reports have suggested that chemotherapy resistant breast tumor cells have a reduced apoptotic index [179] and that the post-chemotherapy apoptotic index correlates with clinical response and increased patient survival [180].

While the promotion of apoptosis may be a desired consequence of chemotherapy and radiation, it is critical to emphasize that other pathways of cell killing as well as interference with proliferative function may have a similar impact on cell survival. In this context, Brown and Wouters cite a number of studies indicating that the lack of correspondence between induction of apoptosis and clonogenic survival in a variety of experimental systems [7]. For instance, p21 wild-type and p21 knockout cells differ in apoptotic susceptibility but demonstrate similar clonogenic survival after exposure to etoposide or irradiation [181]. Bunz et al. have shown that adriamycin induces apoptosis in p53 and p21 knockouts and not in p53 wild-type cells [82]; interestingly, however, while the apoptotic potential was clearly different, there was no evident difference in the response to ionizing radiation in the p53 wild type and p53 mutated tumor cell xenografts [82]. What may be of even greater interest is the demonstration that cell killing and growth inhibition are transient effects; that is, even with extensive cell killing, tumor regrowth occurs after irradiation [184]. Similar findings have been reported in studies of breast tumors in xenograft models [185, 186], particularly where efforts have been made to modulate sensitivity to conventional chemotherapeutic agents with the goal of converting transient inhibition of tumor cell growth to irreversible cell death.

Other studies have demonstrated that while apoptosis may accelerate cell killing by drugs such as VP-16 and adriamycin, ultimate cell survival is not necessarily influenced by the mode of cell death [7, 182, 183]. Consequently, it remains to be established whether the promotion of apoptosis will prove to be effective in enhancing the clinical responsiveness of breast cancer to chemotherapy and radiotherapy. What may prove to be even more relevant is the possibility that the failure of the breast tumor cells to undergo apoptosis in response to radiation or chemotherapy could influence disease recurrence by permitting the survival and regrowth of a sufficient number of tumor cells to repopulate the breast (or other tumor sites to which the cells have metastasized) [187]. This hypothesis is supported by work from ~~the~~ laboratories of Waldman et al. [184] which indicates that cells with intact G1 checkpoint function demonstrate prolonged growth arrest in the absence of apoptosis and that growth arrested tumor cells (studied as xenografts) retain the capacity to recover reproductive capacity. It should be noted that the absence of apoptotic cell death is associated with increased chromosomal instability which can lead to radioresistance [188, 189] and presumably also to chemoresistance. Conversely, tumor cells which undergo apoptotic cell death are, by definition, unable to recover and to repopulate the breast or other tissue sites.

In summary, a variety of cellular signaling pathways may play a role in attenuating or abrogating the apoptotic response to DNA damage in the breast tumor cell. However, the absence of an immediate apoptotic response to radiation or chemotherapeutic drugs such as adriamycin does not eliminate other avenues for cell death and/or loss of reproductive capacity. Conversely, efforts to promote apoptotic cell death in the breast tumor cell could prevent the prolonged growth arrest which may provide an opportunity for subpopulations of breast tumor cells to recover proliferative capacity and to develop resistance to subsequent clinical interventions. Consequently, pharmacological modulation of apoptosis in response to conventional chemotherapy and radiotherapy could prove to be an effective approach for interfering with disease recurrence.

Notes

1. Non-apoptotic cell death followed by prolonged growth arrest in response to acute exposure to 1 μM adriamycin has also been observed in this laboratory with p53 wild-type ZR-75-1 and p53 mutated MDA-MB231 and T-47D breast tumor cells.
2. Recent work in this laboratory indicates that 2–3 days of continuous exposure of MCF-7 cells to 10 μM VM-26 also produces morphological evidence of apoptosis, but only in a limited fraction of the cell population.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 1 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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FOR THE COMMANDER:

PHYLLIS M. RINEHART
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